The Design and Preparation of Pyridoxal 5'-phosphate Analogues

Jasmine Lee

A thesis submitted in partial fulfilment of the requirements of the University of Abertay Dundee for the degree of Doctor of Philosophy

July 2002

I certify that this is the true and accurate version of the thesis approved by the examiners.

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Declaration

I hereby declare that the work presented in this thesis was carried out by me at the University of Abertay Dundee, Dundee, except where due acknowledgement is made, and has not been submitted by me for any other degrees.

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Contents

Acknowledgements

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I would also like to thank the EPSRC for the financial support.

Abstract

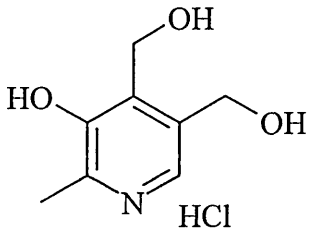
Since the original isolation of pyridoxine **1** (a member of the vitamin B_6 group), many analogues have been designed in an attempt to affect the biological and biochemical systems involving vitamin B_6. The analogues of pyridoxal 5'-phosphate are of particular interest because pyridoxal 5'-phosphate, as a coenzyme, is involved in a number of bio-catalytic reactions (e.g. transamination and decarboxylation of amino acids).

This study involves the design and synthesises of pyridoxal 5'-phosphate analogues using two approaches:

a) The modification of pyridoxine; and

b) Total synthesis via Diels-Alder reaction.

a) The modification of pyridoxine **1** involved using a series of blocking and deblocking procedures. The synthesis of 3,5'-*O*-dibenzylpyridoxine **127** obtained through multiple steps allowed for selective modification of the 4-position of the pyridoxine derivative. Oxidation of **127** afforded the aldehyde derivative **128** in high yield. Subsequent Grignard reaction and hydrolysis afforded the alcohol **241**, which was subjected to oxidation to give the corresponding ketone **242** in high yield.



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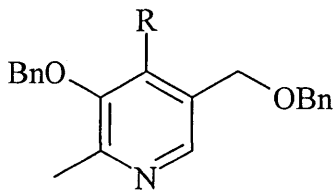


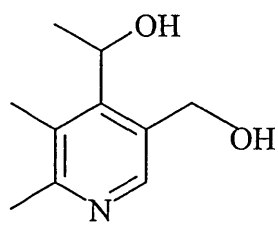
127 R = -CH_2OH

128 R = -CHO

241 R = —C(OH)—CH_3

242 R = —C(=O)—CH_3

The removal of the benzyl groups from compound **241** afforded pyridoxine derivative **243** in 75 % yield.



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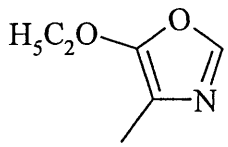
b) The Diels-Alder reaction of oxazole **222** with dienophile, α,β-unsaturated nitrile **223**, was another approach for preparing vitamin B_6 analogues. The Robinson-Gabriel cyclodehydration of α-acylamino carbonyl compound produced the oxazole **222** in good yield. However, difficulties were associated with synthesising the α,β-unsaturated nitrile **223**.



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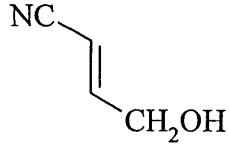

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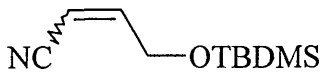
Therefore, addition of protective groups to the β-hydroxy nitrile prior to the formation of α,β-unsaturated nitriles was investigated and did produce a more stable derivative in the form of 4-(*tert*-butyldimethylsilanyloxy)but-2-enenitrile **261** and 4-benzyloxybut-2-enenitrile **268**.



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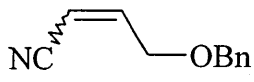

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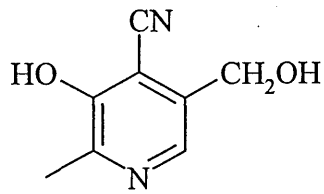
The Diels-Alder reaction of oxazole **222** with commercially available dienophiles, dimethyl maleate and acrylonitrile, was attempted as model reactions. The model reactions afforded dimethyl 5-hydroxypyridine-3,4-dicarboxylate **274** in 36 % yield and 4-cyano-3-hydroxy-2-methylpyridine **276** in 34 % yield. However, Diels-Alder reaction of oxazole **222** with dienophile **268** failed to give the desired pyridine **224** with benzyl protection at C5-hydroxymethyl.



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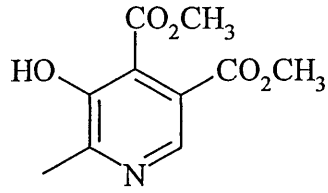

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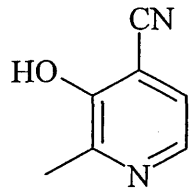

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Abbreviations

Bold Arabic numerals in the text refer to the diagrams of the structural formulae and the Arabic superscripts indicate references. The following abbreviations have been used in the text.

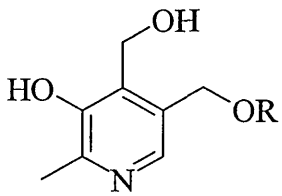
ADP	Adenosine diphosphate
ATCase	Aspartate transcarbamoylase
ATP	Adenosine triphosphate
Bn	$-CH_2C_6H_5$ group (Benzyl)
bp	Boiling point
ca.	Circa
CAD	The multifunctional polypeptide containing the activities of ATCase, CPSase II and DHOase.
$CDCl_3$	Deuterated chloroform
CNS	Central nervous system
CPSase II	Carbamoyl-phosphate synthetase
d	Doublet
dd	Double doublet
dt	Double triplet
DHOase	Dihydroorotase
DMF	*N,N*-Dimethylformamide
DMSO-d_6	Deuterated dimethyl sulphoxide
E	Energy (in eV)
E	*Entgegen* = opposite
EA	Electron affinity
E. coli	*Escherichia coli*
e.g.	For example
Et	$-CH_2CH_3$ group (Ethyl)
FAD	Flavine adenine dinucleotide, oxidised form
FMN	Flavine mononucleotide
FMO	Frontier molecular orbitals
g	grammes
h	Hour(s)
[H]	Reduction
Hb	Haemoglobin
^{1}HNMR (NMR)	Proton nuclear magnetic resonance spectrum
HOMO	Highest occupied molecular orbital

Hz	Hertz
IP	Ionisation potential
IR	Infrared spectrum
J	Coupling constant
L	Leaving group
LUMO	Lowest occupied molecular orbital
m	mutiplet
M^{2+}	Metal ion
MCPBA	*meta*-Chloroperbenzoic acid
Me	$-CH_3$ group (Methyl)
MEM	$-CH_2OC_2H_4OCH_3$ group (2-Methoxyethoxymethyl)
min	Minute(s)
mL	Millilitre(s)
mmol	Millimole(s)
mol	Mole(s)
mp	Melting point
MS	Mass spectrum
Ms	$-S(O)_2CH_3$ group (Mesyl)
N	Normal
NAD^+	Nicotinamide adenine dinucleotide, oxidised form
NADH	Nicotinamide adenine dinucleotide, reduced form
[O]	Oxidation
P	$-PO_3H_2$ group (Phosphates)
4-PA	4-Pyridoxic acid
PDC	Pyridinium dichromate
PG	Protecting group
Ph	$-C_6H_5$ group (Phenyl)
pH	Expressing the acidity or alkalinity of a solution
PL	Pyridoxal
PLP	Pyridoxal 5'-phosphate
PM	Pyridoxamine
PMP	Pyridoxamine 5'-phosphate
PN	Pyridoxine
PNP	Pyridoxine 5'-phosphate
PRPP	5-Phosphoribosyl 1-pyrophosphate
q	Quartet
R	Alkyl group ($-CH_3$, $-C_2H_5$ etc.)
RBC	Red blood cell
s	Singlet

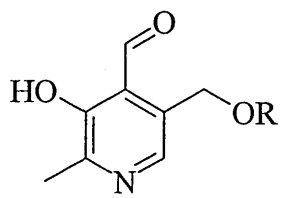
t	Triplet
TBDMS	$-Si(CH_3)_2C(CH_3)_3$ group (*tert*-Butyldimethylsilyl)
THF	Tetrahydrofuran
tlc	Thin layer chromatography
TOSMIC	Tosylmethyl isocyanide
TPP	Thiamine pyrophosphatase
Ts	$-S(O)_2C_6H_4CH_3$ group (Tosyl)
UTP	Uridine triphosphate
UV	Ultraviolet (spectrum)
X	Electron-releasing group
Z	Electron-withdrawing group
Z	*Zusammen* = together
δ	Chemical shift (parts per million)
°C	Degrees celsius
ν	Frequency
cm^{-1}	Wavenumber

1. Introduction

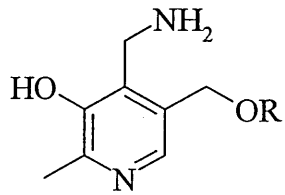
Vitamin B_6 consists of three related pyridine vitamers: pyridoxine **1**, pyridoxal **2**, and pyridoxamine **3**, and their phosphate esters **4**, **5** and **6**. Vitamers are chemical compounds structurally related to a vitamin, and converted to the same overall active metabolites in the body. They thus possess the same kind of biological activity, although sometimes with lower potency. The catalytically active form of vitamin B_6 is pyridoxal 5'-phosphate **5**. The term "vitamin B_6" is used to refer to all 3-hydroxy-2-methyl pyridine derivatives **7** that can mimic the biological activity of pyridoxine.



1 Pyridoxine (PN)
R = H



2 Pyridoxal (PL)
R = H

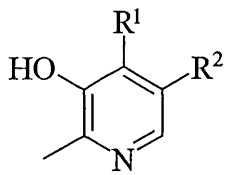


3 Pyridoxamine (PM)
R = H

4 Pyridoxine 5'-phosphate (PNP)
$R = PO_3H_2$

5 Pyridoxal 5'-phosphate (PLP)
$R = PO_3H_2$

6 Pyridoxamine 5'-phosphate (PMP)
$R = PO_3H_2$



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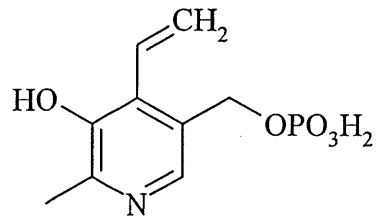
Since the isolation of pyridoxine, many analogues were designed to influence the diverse biological and biochemical systems involving vitamin B_6. In particular, the analogues of pyridoxal 5'-phosphate **5** are of interest because as a coenzyme form of vitamin B_6 it is a constituent of a number of enzymes that catalyse diverse reactions, such as transamination and decarboxylation of amino acids. Thus, the use of coenzyme analogues could provide information on the significance of certain chemical groups in effecting the enzymatic reaction and in binding with the protein moiety. Analogues were utilised in the biochemical area to investigate the substrate specificity and inhibition of enzymes involved in vitamin B_6 metabolism[1]. Also, analogues provided understanding of the structure and synthesis of vitamin B_6, the mode of binding of the coenzyme to the apoenzyme and the structural requirements

for its coenzyme functions. Apoenzyme is the protein component of an enzyme, to which the coenzyme attaches to form an active enzyme. Furthermore, vitamin B_6 analogues revealed the importance of various functional groups in reactions catalysed by pyridoxal in nonenzymatic[2] and enzymatic[3] systems. The involvement of pyridoxal 5'-phosphate analogues in various enzyme systems has offered an opportunity for rational design of inhibitors against various biological systems, such as the 4-vinylpyridoxol 5'-phosphate **8** which inhibits pyridoxine oxidase from rabbit liver[4].



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Furthermore, inhibition of growth in micro-organisms[5] and tumours[6] has been observed with analogues of vitamin B_6. Therefore, analogues containing reactive groups may react irreversibly with enzymes, and thus provide information regarding the nature of active sites.

This study involves the design and preparation of pyridoxal 5'-phosphate analogues, as earlier research has shown that pyridoxal 5'-phosphate does have an effect on the activity of the enzymes involved in pyrimidine biosynthesis in mammalian cells[7]. Thus, pyridoxal 5'-phosphate analogues will act as substrates to develop an understanding of the interaction between the enzyme-substrate complexes in the enzymatic binding sites of pyrimidine biosynthesis in mammalian cells.

The biosynthesis of pyrimidine nucleotides *de novo* is a crucial pathway in growing and dividing cells, and thus the enzymes that catalyse these reactions are of interest in cancer chemotherapy and drug regimens against parasites. In mammalian cells, the three enzyme activities found in the multifunctional polypeptide CAD initiates pyrimidine biosynthesis. The three enzymes are the glutamine-dependent carbamoyl-phosphate synthetase (CPSase II), aspartate transcarbamoylase (ATCase) and dihydroorotase (DHOase). The key enzyme, CPSase II is regulated in the cell negatively by uridine triphosphate (UTP) and positively by 5-phosphoribosyl 1-pyrophosphate (PRPP) which increases the overall synthesis of the carbamoyl-phosphate tenfold at low concentrations of ATP and magnesium ion[8,9]. As

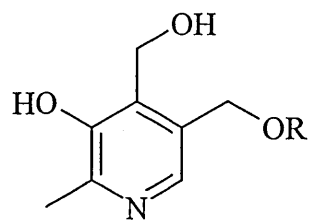
biosynthesis of pyrimidine nucleotides is essential for most growing cells, the application of potential analogues will contribute further understanding on the binding sites of UTP and PRPP in the mammalian multifunctional polypeptide CAD.

Earlier research has shown that UDP-pyridoxal and pyridoxal 5'-phosphate have similar activating activity on the CPSase II suggesting that the effector-binding site is common to the UTP, PRPP and to analogues of these metabolites[7,10]. Thus, designing pyridoxal 5'-phosphate analogues and the application in the study of the mammalian multifunctional polypeptide CAD binding sites will be important for the development of antiproliferative agents. Hence, in this research the pyridoxal 5'-phosphate analogues will be synthesised using two approaches:

a) The modification of commercially available pyridoxine hydrochloride; and
b) The Diels-Alder cycloaddition of substituted oxazole with dienophile.

1.1. Pyridoxine: discovery.

It was first observed in 1926 that rats with vitamin B complex deficiency showed poor growth and development. Even before the general availability of thiamin (vitamin B_1) and riboflavin (vitamin B_2) in pure form, it became evident that the addition of these two vitamins to the diet failed to permit normal growth and development of rats. After several weeks, these animals developed a type of dermatitis (termed acrodynia) characterised by redness and swelling of the tips of the ears, nose and paws which eventually led to necrosis of these parts. It was not until 1934 that György[11] prevented these symptoms by feeding yeast or other sources of the vitamin B complex to rats, and named the acrodynia-preventing factor as vitamin B_6. Within 5 years, vitamin B_6 obtained from rice bran and yeast was isolated and characterised by several laboratories[12,13] as 3-hydroxy-4,5-dihydroxymethyl-2-methyl pyridine **1** (subsequently named as pyridoxine).



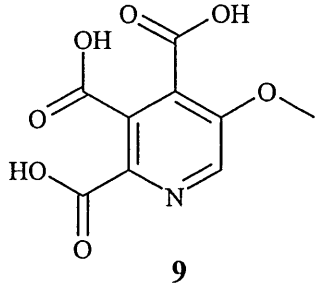
1 R = H

1.1.2. The structural elucidation of pyridoxine.

Pyridoxine **1** behaves as a weak base and when treated with diazomethane it forms monomethyl ether, which, on acetylation, gives a diacetyl derivative. It therefore appears that the three oxygen atoms in pyridoxine are present as hydroxyl groups, and since one is readily methylated, this one is probably phenolic. This is supported by the fact that pyridoxine produces the ferric chloride colour reaction of phenols. Thus, the other two hydroxyl groups are alcohols.

Pyridoxine's ultraviolet absorption spectrum was shown to be similar to that of 3-hydroxypyridine. This similarity gives the indication that pyridoxine is a pyridine derivative with the phenolic group in the 3-position. Since lead tetra-acetate has no action on the monomethyl ether of pyridoxine, this leads to state that the two alcoholic groups are not on adjacent carbon atoms in a side-chain[13]. When this methyl ether is carefully oxidised with alkaline potassium permanganate, the methoxypyridinetricarboxylic acid ($C_9H_7NO_7$) was produced which gave a blood-red colour with ferrous sulphate (a characteristic reaction of pyridine-2-carboxylic acid). Therefore, one of three carboxyl groups is in the 2-position.

When the methyl ether of pyridoxine was oxidised with alkaline permanganate under the usual conditions, the products were carbon dioxide and the anhydride of a dicarboxylic acid ($C_8H_5NO_4$), this indicates that these two carboxyl groups are in the *ortho*-position. This anhydride, on hydrolysis to its corresponding acid, did not give a blood-red colour with ferrous sulphate, thus confirming the absence of a carboxyl group in the 2-position. It therefore follows that, on decarboxylation, the tricarboxylic acid eliminates the 2-carboxyl group to form the anhydride. The tricarboxylic acid could have been either structure **9** or **10**.



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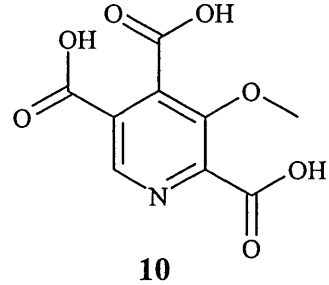

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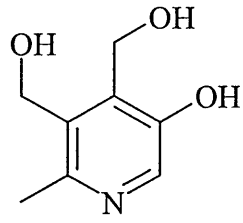
As pyridoxine methyl ether contains three oxygen atoms (one as methoxyl and the other two alcohols), it is possible that two carboxyl groups in the tricarboxylic acid

could arise from two CH_2OH groups, and the third from a methyl group. Therefore, the pyridoxine could be either **11** or **12**.



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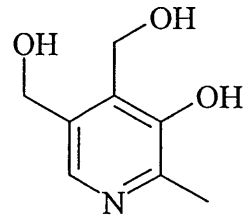

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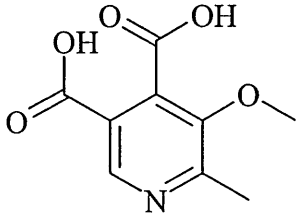

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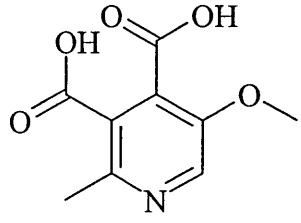

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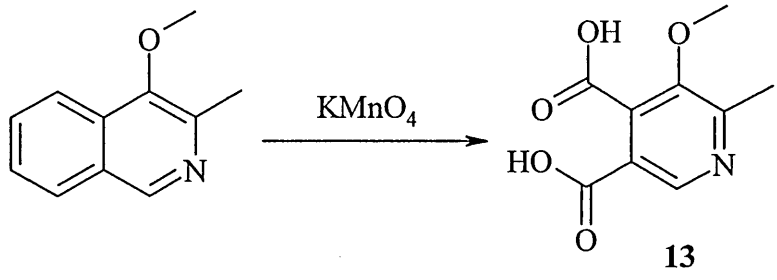
The structure of pyridoxine was definitively determined when pyridoxine methyl ether was oxidised with barium permanganate to afford a dicarboxylic acid ($C_9H_9NO_5$), which did not give a red colour with ferrous sulphate. Thus, there is no carboxyl group in the 2-position. Also, since the dicarboxylic acid readily formed an anhydride and gave a phthalein on fusion with resorcinol, the two carboxyl groups must be in the *ortho*-position. The analysis of both the dicarboxylic acid and its anhydride showed the presence of a methyl group; thus, the structure of the dicarboxylic acid could be **13** or **14**. Eventually, it was shown that the anhydride was **13** from its formation by the oxidation of 4-methoxy-3-methylisoquinoline[13] (scheme 1).



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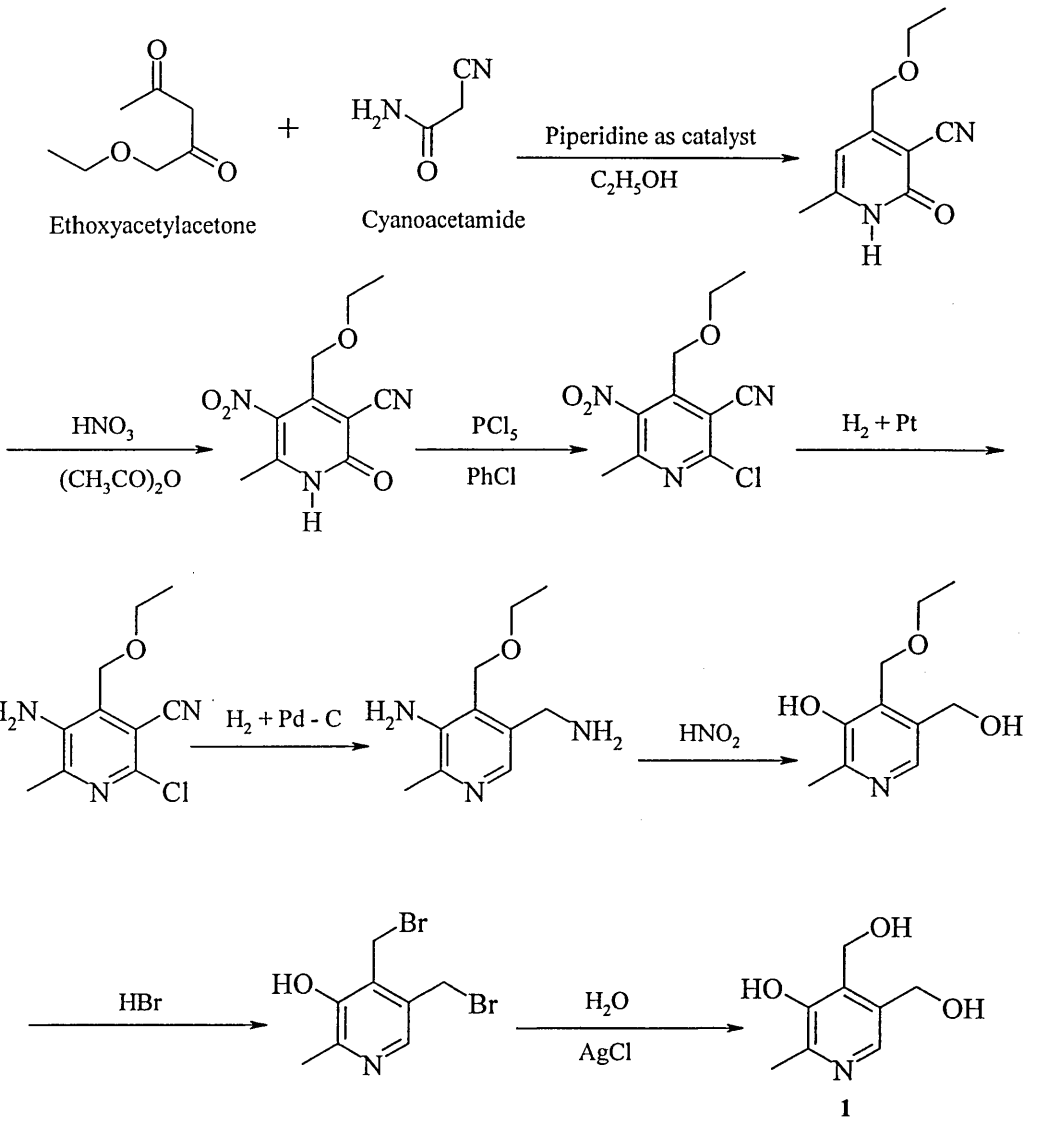
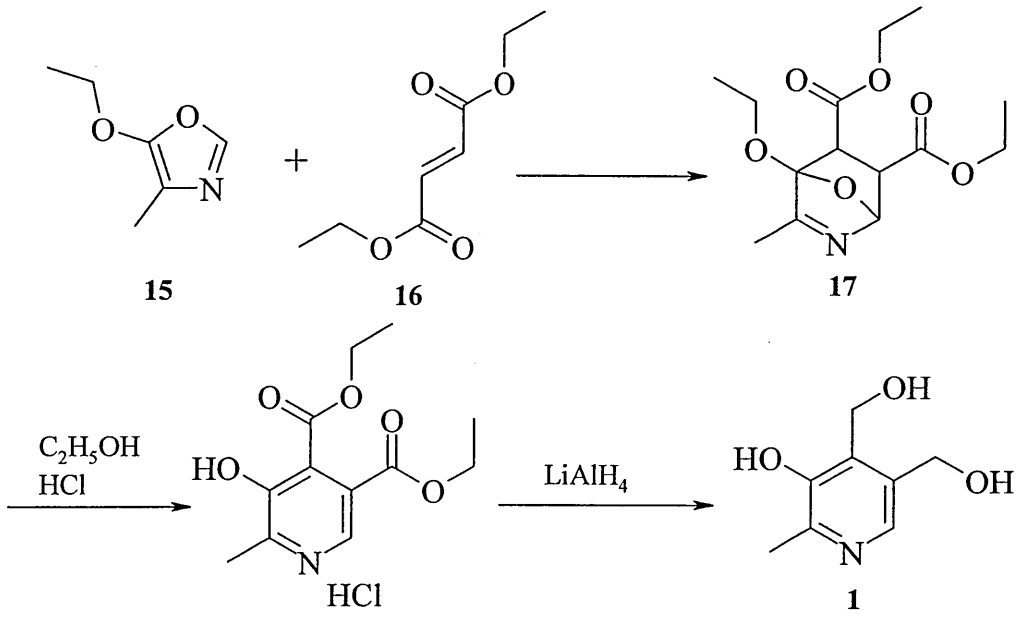
Scheme 1

In addition, the structural elucidation of pyridoxine **1** (3-hydroxy-4,5-dihydroxymethyl-2-methyl pyridine) was confirmed by synthesis. For example, Harris and Folker[14] used acyclic precursors, cyanoacetamide and ethoxyacetylacetone, to produce pyridoxine **1** as shown in scheme 2:



Scheme 2

Another synthesis[15,16] involves Diels-Alder reaction of 5-ethoxy-4-methyloxazole **15** with diethyl maleate **16** to give **17**, which after treatment with acid followed by reduction with lithium aluminium hydride was converted into pyridoxine **1** (scheme 3).

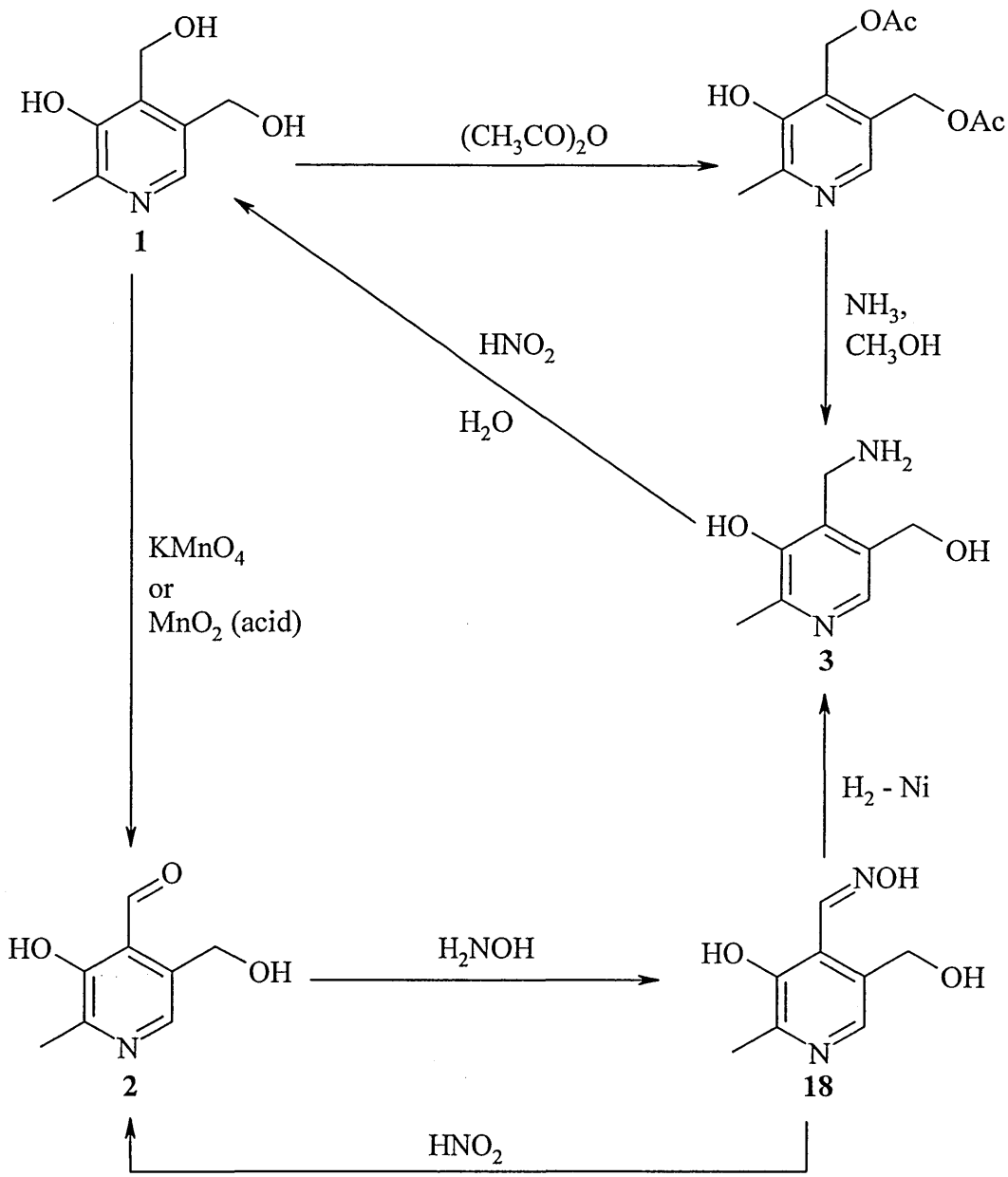


Scheme 3

1.1.3. The vitamin B_6 group.

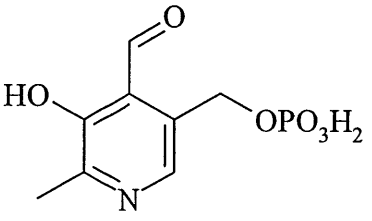
Although pyridoxine **1** has vitamin activity, it was revealed that 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine (pyridoxal **2**) and 2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine (pyridoxamine **3**) also contributed to the vitamin B_6 activity in animals and lactic acid bacteria[17,18]. Observation in rats revealed that ingested pyridoxine **1** was converted to compounds **2** and **3**. Pyridoxal and pyridoxamine have been shown subsequently to comprise most of the vitamin B_6 in natural materials[19]. These three compounds are interchangeable and approximately equally active in supporting growth of rats, dog, and chicks fed with vitamin B_6 deficient rations and in supporting growth of vitamin B_6 dependent fungi and some bacteria. In synthesis, pyridoxine **1** could be converted to compound **2** or **3** by partial oxidation or by amination to yield an aldehyde or amine, respectively[20]. The inter-relationship between pyridoxine **1**, pyridoxal **2** and pyridoxamine **3** was established by transformations (scheme 4)[20]. In scheme 4, careful oxidation of pyridoxine **1** with potassium permanganate isolated pyridoxal **2** as its oxime **18**. Treatment of oxime **18** with nitrous acid gives pyridoxal **2**, whereas catalytic reduction forms pyridoxamine **3**. Pyridoxamine **3** was treated with nitrous acid to form pyridoxine **1**, and this can be acetylated followed by amination to regenerate pyridoxamine. Eventually, pyridoxine **1**, pyridoxal **2**, pyridoxamine **3**, and all 3-hydroxy-2-methyl pyridine derivatives that

can mimic the biological activity of pyridoxine were collectively referred to as 'vitamin B_6'.



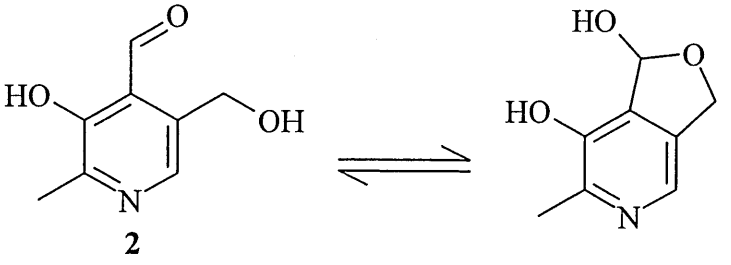
Scheme 4

Most of the vitamin B_6 in natural materials are present as phosphorylated derivatives of compounds **1**, **2**, and **3**. In 1944, a compound required for enzymatic decarboxylation of amino acids was isolated from yeast[21]. It was subsequently shown that the compound was pyridoxal-5-phosphate **5** (also referred to as codecarboxylase) through comparison with the synthetic material prepared in low yield by phosphorylation of pyridoxal using phosphoryl chloride in the presence of water[22].



5

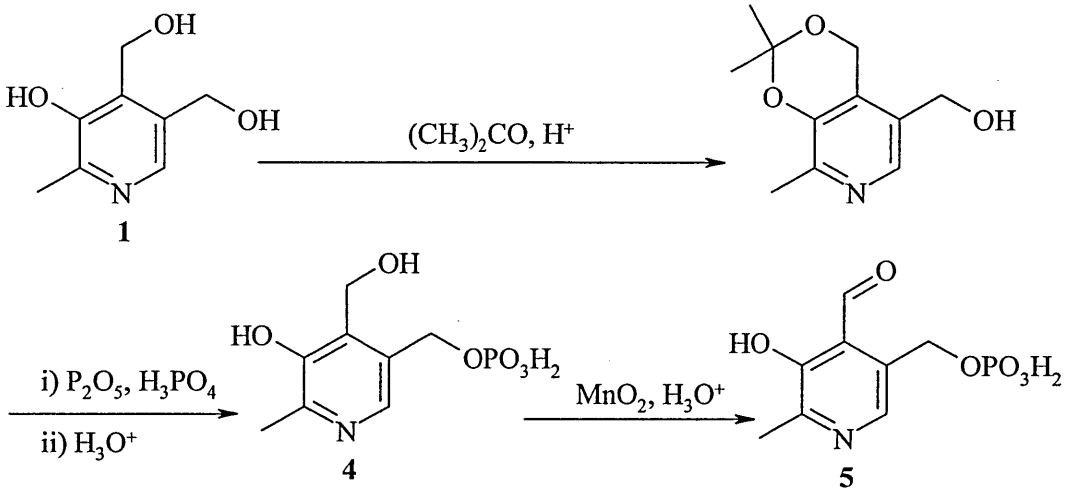
The low yield of the phosphorylated derivative is due, presumably, to the fact that pyridoxal **2** exists largely in its cyclic hemiacetal form (scheme 5).



2

Scheme 5

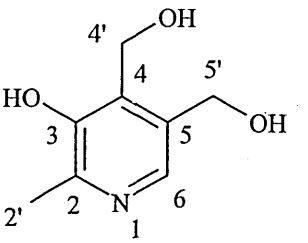
A better preparative route lies in phosphorylation of pyridoxamine **3** to yield pyridoxamine-5-phosphate **6**, which is readily oxidised to pyridoxal-5-phosphate **5** with manganese dioxide[23,24]. The precise location of the phosphate residue was established by an elimination procedure and the structure **5** was eventually confirmed by the unambiguous synthesis[25] set out in scheme 6. Vitamin B_6 in the form of their phosphates is inter-convertible in the body, and has been shown that the aldehyde and the amine are the main constituents.



Scheme 6

1.2. Biosynthesis of pyridoxine.

De novo biosynthesis of pyridoxine (vitamin B_6) is a process peculiar to micro-organisms and attempts to establish the nature of precursors, intermediates, and biosynthetic pathways were complicated by the very small quantities of the vitamin produced by most organisms. Since the 1960s, the pathway leading to pyridoxine **1** synthesis has been largely defined in *Escherichia coli* through the study of tracer experiments using radio-labelled precursors and pyridoxine auxotrophic mutants.

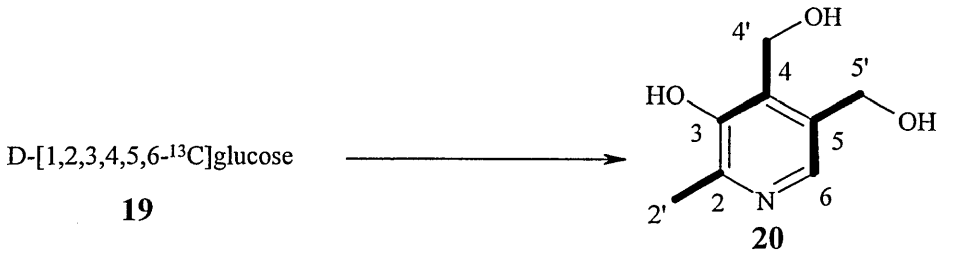


1

Tracer experiments have provided an understanding of the biogenetic anatomy of the pyridoxine skeleton, in terms of its derivation from glucose, glycerol, and several other primary metabolites[26]. Most of the tracer studies were performed with cultures of *E. coli* mutant **WG2**, which lacks the enzyme pyridoxine 5'-phosphate oxidase[27]. This mutant was used as it synthesised pyridoxine and its 5'-phosphate ester at a rate that is four or five times that of the wild-type[28]. Early studies[29,30]exploited non-randomly ^{14}C-labelled substrates as tracers and ^{3}H were used on occasion (as a secondary tracer) in conjunction with ^{14}C as an internal standard. In recent studies[31,32] stable isotopes such as ^{13}C or ^{2}H were employed. In particular, the application of substrates that are fully ^{13}C enriched at contiguous carbon atoms[31,33] (called 'bond-labelled' samples) enables the observation of intact multicarbon units transferred from precursor into biosynthetic product.

1.2.1. Glucose as the primary precursor.

In an experiment with D-[1,2,3,4,5,6-^{13}C]glucose **19**, the ^{13}C NMR spectrum of the isolated sample of pyridoxine **20** demonstrated that only two carbon-carbon bonds, those between C-2 and C-3 and between C-4 and C-5, are newly formed in the course of the biosynthetic derivation of pyridoxine from glucose[31]. Hence, glucose supplied three intact multicarbon units, as the building blocks of the three fragments, C-2,2', C-3,4,4', and C-6,5,5', of pyridoxine (scheme 7).



Scheme 7

Although the experiment identified that glucose supplies three multicarbon units and the sites into which these are introduced, however, it does not provide evidence concerning the following:

(i) The identity of the 3 C and 2 C intermediates.

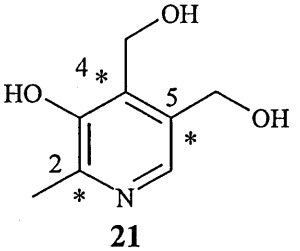
(ii) The fragments of glucose that supply them.

(iii) The sequence of events whereby these fragments are generated.

(iv) The sequence and the mechanism whereby these fragments combined to yield the pyridoxine skeleton.

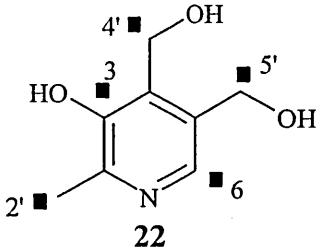
The probable identity of the glucose-derived intermediates that serve as the precursors of the 3 C and 2 C fragments was provided from the results of experiments with ^{14}C-labelled samples of glucose, glycerol, and pyruvic acid. It had been shown[29] that the 3 C and 2 C units in the carbon skeleton of pyridoxine **20** to be derived from glycerol in a very specific manner. The chemical degradation of the pyridoxine sample **21** isolated from an experiment using [2-^{14}C]glycerol revealed that radioactivity was distributed equally over three sites, C-2, C-4, and C-5, of the vitamin (33 % of total label of pyridoxine at each of three sites). The companion experiment with [1,3-^{14}C]glycerol displayed 20 % of total label of pyridoxine at each of the location of C-2', C-4', and C-5'. Also, the ^{13}C NMR spectrum of the pyridoxine sample **22** that was isolated from the incubation with [1,3-^{13}C]glycerol demonstrated signals due to C-2', C-3, C-4', C-5', and C-6 with each showing approximately 20 % enrichment.

Labelling patterns within samples of pyridoxine derived from various labelled substrates.



21

* [2-^{14}C]glycerol

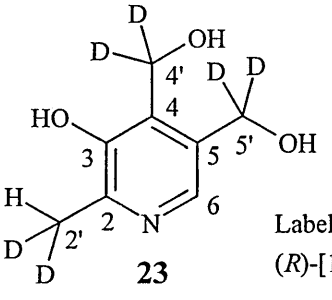


22

■ [1,3-^{13}C]glycerol

Thus, when glycerol serves as the carbon source, five of the eight carbon atoms of pyridoxine (C-2', C-3, C-4', C-5', and C-6) are derived from its primary carbon atoms and three (C-2, C-4, and C-5) from its secondary carbon. The 8 C skeleton of pyridoxine must be constructed from three glycerol units, one of which loses a primary carbon on the route to the product.

In an experiment employing $^{3}H/^{14}C$ double labelling[34], it was demonstrated that six of the eight hydrogen atoms of pyridoxine are represented by hydrogen atoms directly derived from the methylene hydrogen atoms of glycerol. It was shown by chemical degradation[35] that the hydrogen atom at C-6 of pyridoxine did not retain a tritium label. As the two terminal CH_2OH groups of glycerol are stereochemically distinct, one or the other of the two terminal groups can be labelled to generate chiral samples. The enantiomeric compounds (*S*)-[1,1-^{2}H]- and (*R*)-[1,1-^{2}H]-glycerol was utilised to observe their mode of incorporation into pyridoxine[35]. In an experiment using the (*R*)-[1,1-^{2}H]-glycerol, the site of the six glycerol-derived hydrogen atoms was determined. The glycerol deuterium atoms were retained within pyridoxine sample **23** in pairs located at the CH_2OH group, C-4', another at the CH_2OH group, C-5', and the third representing two of the three hydrogen atoms at the C-methyl group, C-2'. Thus, the three pairs of hydrogen atoms retained at C-2', C-4', and C-5' were derived from the (*R*)-terminus of glycerol.



23

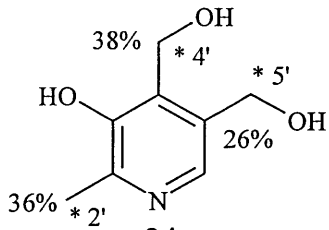
Labelling patterns of pyridoxine sample **23** derived from (*R*)-[1,1-^{2}H]glycerol.

The (*S*)-terminus of glycerol did not contribute deuterium to the biosynthetically generated pyridoxine. Thus, the findings illustrate that glycerol enters each of the three subunits of pyridoxine in a stereospecific manner, in which the intact carbon chain of glycerol (*pro-R*)CH_2(OH)-CH(OH)-(*pro-S*)CH_2OH would yield the carbon atoms C-4',4,3 and C-5',5,6, respectively, of each of the two 3 C units. Finally, the C-2',2 unit originates from the (*pro-R*)CH_2(OH)-CH(OH)- unit of glycerol.

Glycerol itself lacks the chemical and biochemical reactivity that is required to create the vitamin skeleton. It is more likely that the reactive species is one or both of the two triose phosphates, dihydroxyacetone 1-phosphate and D-glyceraldehyde 3-

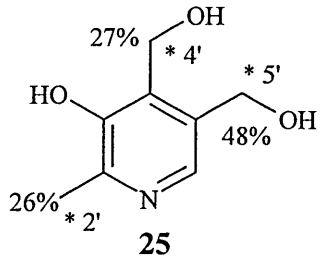
phosphate, that are generated from glycerol in the course of its metabolism, and that the intact units, C-3,4,4' and C-6,5,5', of pyridoxine are derived from them. When glycerol is phosphorylated on its route into the triose phosphates, only the *pro-R* hydroxymethyl group is phosphorylated[36]. Thus, if glycerol does indeed enter pyridoxine by way of these triose phosphates, the prochiral carbon atoms of glycerol must be incorporated into the vitamin in a regiospecific manner. The suggestion of triose phosphates as intermediates in pyridoxine biosynthesis came from the experiments[29] with [1-^{14}C]glucose and [6-^{14}C]glucose isolated pyridoxine samples **24** and **25**, respectively. Glycolytic breakdown of each of [1-^{14}C]glucose and [6-^{14}C]glucose yields triose phosphates that are singly labelled at the terminal carbon that carries the phosphate group. Incorporation of these labelled triose phosphates derived from glucose would expect to deliver the label into each of the three sites that are derived from the *pro-R* hydroxymethyl group of glycerol, that is C-2', C-4', and C-5'. Hence, the observation from the chemical degradation of the pyridoxine samples from the two experiments showed, in each case, that labels at C-2', C-4', and C-5' accounted for all the radioactivity within the two samples of the vitamin that were derived from [1-^{14}C]glucose and from [6-^{14}C]glucose.

Labelling patterns of pyridoxine samples derived from labelled glucose.



24

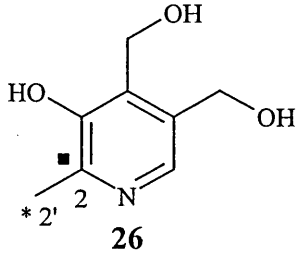
* [1-^{14}C]glucose



25

* [6-^{14}C]glucose

The source of the C-2',2 unit was identified as the CH_3CO fragment arising by decarboxylation of pyruvic acid[29]. Tracer experiments with pyruvate revealed that the carboxyl group was lost and that the CH_3CO unit gave rise to C-2',2 of pyridoxine sample **26**.

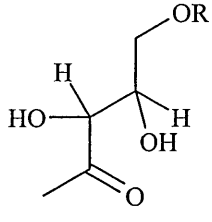


26

Labelled pyridoxine sample **26** derived from

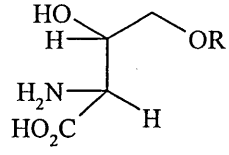
* [3-^{14}C]pyruvic acid and ■ [2-^{14}C]pyruvic acid.

These results are consistent with the generation of the C-2',2 of pyridoxine from glycerol, via triose phosphate. It is the *pro-S* hydroxymethyl group of glycerol that is transformed into the aldehyde group of D-glyceraldehyde 3-phosphate and then into the carboxylic acid group of pyruvic acid, which is lost. It is likely that the C-2',2 of pyridoxine is not generated by direct entry of a two-carbon precursor. Instead, the removal of the carboxyl group of pyruvic acid takes place at an earlier stage, in the course of the synthesis of a 5 C intermediate. Therefore, the 5 C unit must be derived from the CH_3CO fragment of pyruvate plus a triose phosphate. The reaction of a triose phosphate, D-glyceraldehyde 3-phosphate (or of D-glyceraldehyde itself), with pyruvic acid, catalysed by pyruvate dehydrogenase, occurs in many micro-organisms, including *E. coli*[37,38]. The reaction is accompanied by loss of the pyruvic acid carboxyl group, to yield a 5 C compound, 1-deoxy-D-xylulose 5-phosphate **27** (or 1-deoxy-D-xylulose **28**, respectively). As tracer experiments revealed that the 5 C compound, 1-deoxy-D-xylulose, serves as an intermediate on the route from glucose into pyridoxine, thus the remaining 3 C, N unit was derived from 4-hydroxy-L-threonine 4-phosphate **29** (or 4-hydroxy-L-threonine **30**, respectively).



27 R = PO_3H_2
1-Deoxy-D-xylulose
5-phosphate

28 R = H
1-Deoxy-D-xylulose



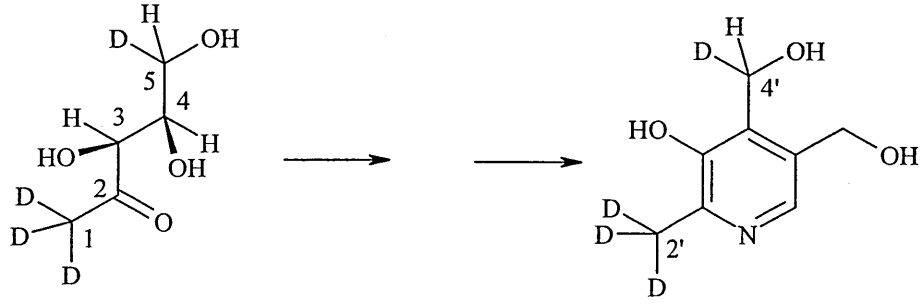
29 R = PO_3H_2
4-Hydroxy-L-threonine
4-phosphate

30 R = H
4-Hydroxy-L-threonine

1.2.2. 1-Deoxy-D-xylulose and 4-hydroxy-L-threonine as the acyclic precursors.

Indications that 1-deoxy-D-xylulose **28** serves as the precursor of the C-2',2,3,4,4' fragment of pyridoxine were observed in the experiments using 1-deoxy[1,1,1-^{2}H,(*RS*)-5-^{2}H]-D-xylulose **31** and 1-deoxy[1,1,1-^{2}H,(*RS*)-5-^{2}H]-L-xylulose samples administrated to mutant *E. coli*[32]. Deuterium NMR spectroscopy revealed that only the D isomer was incorporated into pyridoxine. Pyridoxine from

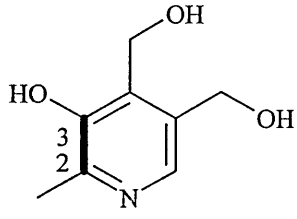
the experiment with the labelled 1-deoxy-D-xylulose contained deuterium at C-2' and at C-4', and the ratio of deuterium at these two sites corresponded to the ratio of deuterium at C-1 and C-5 of the substrate (scheme 8)[32].



31 1-Deoxy[1,1,1-^{2}H,(*RS*)-5-^{2}H]-D-xylulose

Scheme 8

Another tracer experiment observed that the presence of unlabelled 1-deoxy-D-xylulose partially inhibited the incorporation of ^{13}C from D-[1,2,3,4,5,6-^{13}C]glucose into C-2',2,3,4,4', but not into C-6,5,5' of pyridoxine[39]. These inferences supported 1-deoxy-D-xylulose as a basic building block of the C-2',2,3,4,4' fragment of pyridoxine in *E. coli.* Additional evidence that the compound supplies the 5 C fragment as an intact unit came from the experiment with [2,3-^{13}C]-1-deoxy-D-xylulose which revealed that the bond was labelled at C-2-C-3 of pyridoxine sample **32**, proving incorporation of the substrate without cleavage of its C-2,3 bond[40].

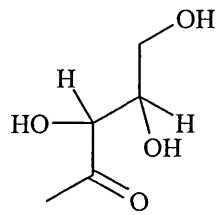


32

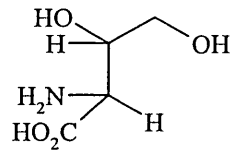
Labelled pyridoxine sample **32**
derived from [2,3-^{13}C]-1-deoxy-D-xylulose.

The participation of 1-deoxy-D-xylulose in pyridoxine biosynthesis has also been examined in a nonbacterial system, using protein fraction derived from spinach chloroplasts[41]. When this soluble protein fraction was incubated in the presence of glyceraldehyde 3-phosphate, pyruvate, glycine, ATP, and Mg^{2+}, vitamin B_6 biosynthesis took place but biosynthesis was inhibited by omission of any one of these substrates. In the absence of pyruvate, biosynthesis was restored by the addition of 1-deoxy-D-xylulose. These results inferred the view that 1-deoxy-D-xylulose **28** is implicated as a precursor of the C-2',2,3,4,4' fragment in pyridoxine biosynthesis. It

is of interest that 1-deoxy-D-xylulose has been implicated also in the biosynthesis of thiamin in *E. coli*[37,38].

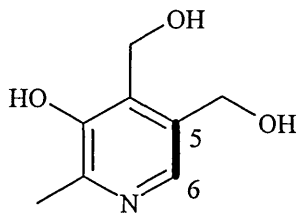


28 1-Deoxy-D-xylulose



30 4-Hydroxy-L-threonine

The remaining segment of pyridoxine, N-1,C-6,5,5', is provided by 4-hydroxy-L-threonine **30**. The pyridoxine sample **33** isolated from the experiment with [2,3-^{13}C]-4-hydroxy-L-threonine revealed that the C-6-C-5 was labelled[33]. Furthermore, the presence of unlabelled 4-hydroxy-L-threonine totally suppressed the incorporation of ^{13}C from D-[1,2,3,4,5,6-^{13}C]glucose into C-6,5,5' but not into C-2',2,3,4,4' of pyridoxine. 4-Hydroxy-L-threonine is thereby shown to lie on the route from glucose into the C-6,5,5' unit of pyridoxine.



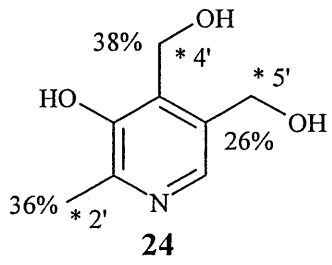
33

Labelled pyridoxine sample **33** derived from [2,3-^{13}C]-4-hydroxy-L-threonine.

1.2.3. Metabolic steps leading from glucose to 1-deoxy-D-xylulose and to 4-hydroxy-L-threonine.

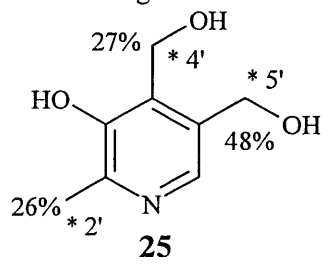
Pyridoxine samples **24** and **25** isolated from the tracer experiments[29] with [1-^{14}C]glucose and [6-^{14}C]glucose, respectively, contains all their radioactivity at the C-2', C-4' and C-5'. However, the three centres were not equally labelled. In the case of pyridoxine sample **24** derived from [1-^{14}C]glucose, the C-5' contained 26 ± 2 % of the total specific activity, while C-2' and C-4' each contained a higher level (36 ± 1 % and 38 ± 4 %, respectively). Conversely, in the case of pyridoxine sample **25** derived from [6-^{14}C]glucose, half (48 ± 4 %) of the label resided at C-5', while C-2' and C-4' each contained only one quarter of the total activity (26 ± 2 % and 27 ± 4 %, respectively). In both instances, the level of specific activity at C-2' and C-4' was similar, but different from that at C-5'.

Labelling patterns of pyridoxine derived from labelled glucose.



24

* [1-^{14}C]glucose



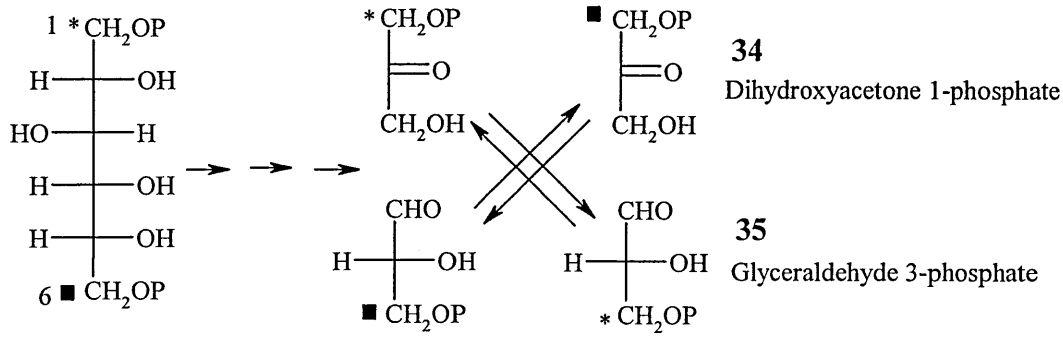
25

* [6-^{14}C]glucose

Therefore, the C-6,5,5' unit of pyridoxine could have derived from glucose in a different manner than the C-3,4,4' unit and that the C-2,2' unit shares its origin with the latter 3 C unit (presumably derived from it). Two different interpretations have been placed on these inferences, in terms of the known processes of the primary metabolism of glucose. One interpretation was based on the assumption that all three fragments were derived by the glycolytic route[29]. Another, more recent, interpretation suggested that only the C-3,4,4' unit and consequently the C-2,2' unit are of glycolytic origin, whereas the C-6,5,5' unit is a product of the pentose phosphate pathway[42]. Genetic evidence strongly supports the latter interpretation[42,43,44].

1.2.4. The origin of 1-deoxy-D-xylulose.

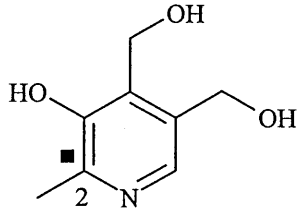
If all three fragments are derived by the glycolytic route, then the glycolytic cleavage of [1-^{14}C]fructose 1,6-biphosphate, derived from [1-^{14}C]glucose, yields [^{14}C]dihydroxyacetone 1-phosphate **34** and unlabelled glyceraldehyde 3-phosphate **35**, the latter which receives the label later by triose phosphate isomerase-catalysed equilibration. In the case of [6-^{14}C]glucose, it is glyceraldehyde 3-phosphate **35** that is labelled first followed by the dihydroxyacetone 1-phosphate **34** (scheme 9). Thus, the two subunits C-2',2 and C-3,4,4', which were equally and more highly labelled (at C-2' and C-4') in pyridoxine sample **24** isolated from an experiment with [1-^{14}C]glucose, must both be derived from C-1,2,3 of glucose via dihydroxyacetone 1-phosphate. On the other hand, the C-6,5,5' unit which receives a larger fraction of label in pyridoxine sample **25** isolated from an experiment with [6-^{14}C]glucose than in pyridoxine sample **24** isolated from an experiment with [1-^{14}C]glucose, is derived from C-4,5,6 of glucose via glyceraldehyde 3-phosphate.



Equilibration of label from C-1 to C-6 of glucose.

Scheme 9

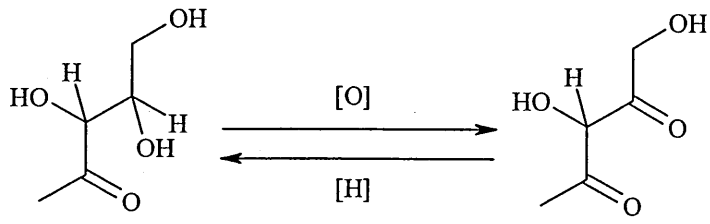
This means a route to pyruvic acid from dihydroxyacetone 1-phosphate is required, as the normal glycolytic route to pyruvate proceeds via glyceraldehyde 3-phosphate. Such a route, via pyruvaldehyde and D-lactate, does exist in *E. coli*[45,46,47]. The observation that [2-^{14}C]pyruvaldehyde yielded pyridoxine sample **36** that was labelled exclusively at C-2 was taken as evidence for the involvement of this route in pyridoxine biosynthesis[34].



36

Labelled pyridoxine sample **36** derived from ■ [2-^{14}C]pyruvaldehyde.

If dihydroxyacetone 1-phosphate **34** is indeed the precursor of the C-3,4,4' unit then the formation of the acyclic precursor of the C-2',2,3,4,4' unit of pyridoxine must take place by condensation of dihydroxyacetone 1-phosphate with pyruvate-derived acetylthiamin pyrophosphate[48], followed by hydrolysis. This would yield the (*R*)-1-deoxy-2,4-dioxopentane-3,5-diol **37** which is the C-4 dehydrogenation product of 1-deoxy-D-xylulose **28**[49]. Since these two 5 C compounds are interconvertible by oxidation/reduction, either one or the other can serve as intermediate precursor of the 5 C fragment of pyridoxine (scheme 10).



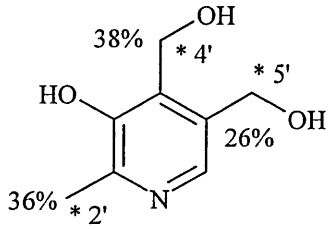
1-Deoxy-D-xylulose **28**

(*R*)-1-Deoxy-2,4-dioxopentane-3,5-diol **37**

Scheme 10

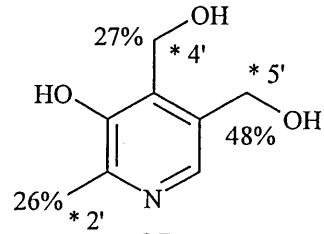
The other interpretation was that the C-6,5,5' of pyridoxine is derived from glucose by way of the pentose phosphate route, whereas the C-3,4,4' originates via glycolytic intermediates. The uneven distribution of label in the pyridoxine samples **24** and **25** derived from [1-^{14}C]glucose and [6-^{14}C]glucose respectively into C-5', on the one hand, and into C-2' and C-4' of pyridoxine, on the other, suggests that the C-5' unit is derived from the pentose route.

Labelling patterns of pyridoxine derived from labelled glucose.



24

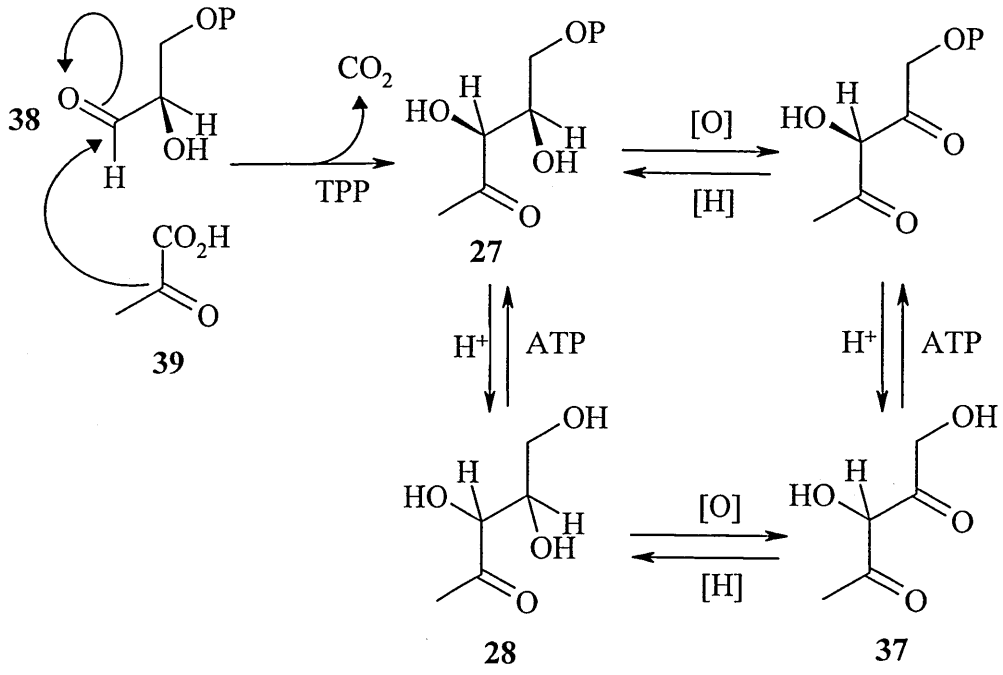
* [1-^{14}C]glucose



25

* [6-^{14}C]glucose

This lead to the assumption that the C-3,4,4' unit could originate from glyceraldehyde 3-phosphate. Also the pyruvic acid, the precursor of the C-2,2' unit, is derived from glyceraldehyde 3-phosphate in the normal course of glycolysis. Hence, the condensation of D-glyceraldehyde 3-phosphate **38** with pyruvic acid **39** are catalysed by the pyruvate dehydrogenase, and accompanied by decarboxylation yields 1-deoxy-D-xylulose 5-phosphate **27** (scheme 11)[50]. This enzymic process, which occurs in *E. coli*[51], requires thiamine pyrophosphatase as a coenzyme. 1-Deoxy-D-xylulose **28** is derivable from **27** by phosphatase-catalysed hydrolysis and convertible into it by kinase catalysed phosphorylation.

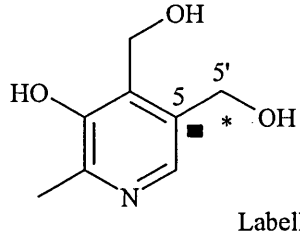


Biosynthesis of 1-deoxy-D-xylulose **28**.

Scheme 11

1.2.5. The origin of 4-hydroxy-L-threonine.

If all three fragments are of glycolytic origin, the 3 C unit, C-6,5,5', of pyridoxine is derived intact from C-4,5,6 of glucose via D-glyceraldehyde 3-phosphate. However, another source of two of these carbon atoms, C-5,5', was discovered in another *E. coli* mutant (mutant **WG3**). This mutant is a pyridoxine-requiring strain, which can utilise glycolaldehyde to satisfy its pyridoxine requirement[52]. Labelled glycolaldehyde was incorporated into pyridoxal 5'-phosphate isolated from this organism[53]. Furthermore, isolated pyridoxine sample **40** revealed the sites of incorporation of the two carbon atoms of glycolaldehyde were shown to be the carbon atom of the CH_2OH group, and the C-5 from the aldehyde carbon atom.



40

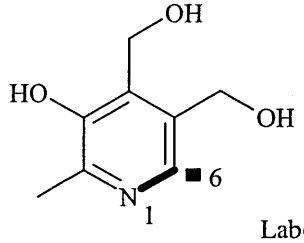
Labelled pyridoxine sample **40** derived from

■ [1-^{14}C]glycolaldehyde and

* [2-^{14}C]glycolaldehyde.

It was shown subsequently that the same process occurred also in the *E. coli* mutant **WG2**, which lacks the pyridoxine 5'-phosphate oxidase, as a minor pathway contributing to the formation of the C-5,5' of pyridoxine, but its major source is a component of the C-6,5,5' fragment which is derived as an intact unit from glucose[54].

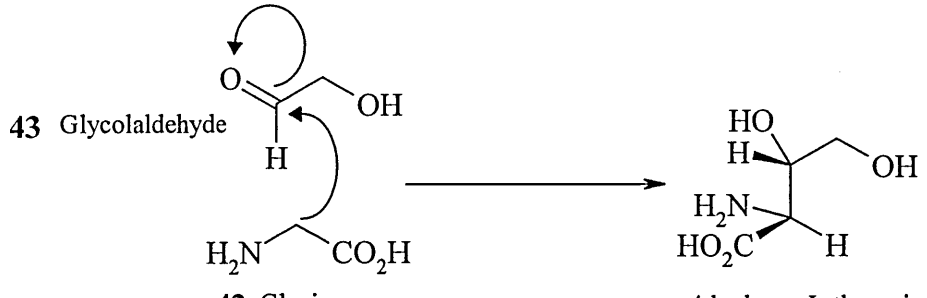
That the two processes were entirely distinct from one another was shown by the fact that whereas C-6 of pyridoxine, in mutant **WG2**, originates from a terminal carbon atom of glycerol when glycerol is the sole primary precursor of the vitamin, the same carbon atom arises from the central carbon atom of glycerol, when C-5,5' originates from glycolaldehyde[54]. This contradiction was explained by the finding that, in mutant **WG3**, C-6 of pyridoxine sample **41** was supplied by the methylene carbon of [2-^{14}C]glycine and that the N-1,C-6 fragment arises from glycine as an intact unit[55]. It was shown by ^{13}CNMR spectroscopy that pyridoxine sample **41**, isolated from a culture of mutant **WG3**, which had been incubated with bond-labelled glycine, $^{15}NH_2$-$^{13}CH_2CO_2H$, maintained the intact ^{15}N-^{13}C bond of the substrate at N-1,C-6[55].



41

Labelled pyridoxine sample **41** derived from
■ [2-^{14}C]glycine and
—— [2-^{13}C,^{15}N]glycine.

Therefore, 4-hydroxy-L-threonine **30** generated by condensation of glycine **42** with glycolaldehyde **43** (scheme 12), in a reaction analogous to that catalysed by threonine aldolase or serine hydroxymethylase, might be an intermediate in pyridoxine biosynthesis serving as the precursor of the N-1,C-6,5,5' unit.



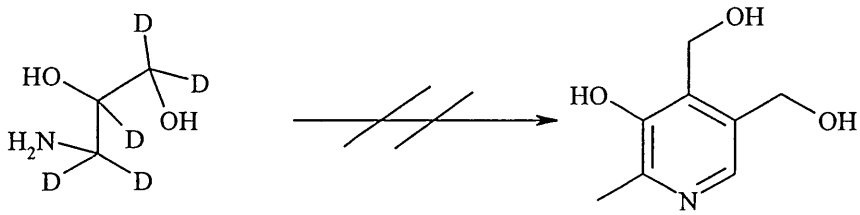
43 Glycolaldehyde

42 Glycine

4-hydroxy-L-threonine
30

Scheme 12

In an attempt to rationalise this duality of origin of the N-1,C-6,5,5' of pyridoxine, the 1-aminopropane-2,3-diol (or its phosphate) was postulated to serve as the ultimate intermediate on the route into the N-1,C-6,5,5' fragment of pyridoxine[26]. (*S*)-1-Aminopropane-2,3-diol is the decarboxylation product of 4-hydroxy-L-threonine, but it may also be generated by transamination of D-glyceraldehyde 3-phosphate followed by phosphate ester hydrolysis. If served as an intermediate, the dual origin of the 3 C, N unit either directly from D-glyceraldehyde 3-phosphate, or from glycolaldehyde plus glycine via 4-hydroxy-L-threonine, would be explicable. The attempt to support this notion experimentally failed, as the label from a ^{2}H-labelled sample of 1-aminopropane-2,3-diol **44** was not incorporated into pyridoxine (scheme 13)[56].

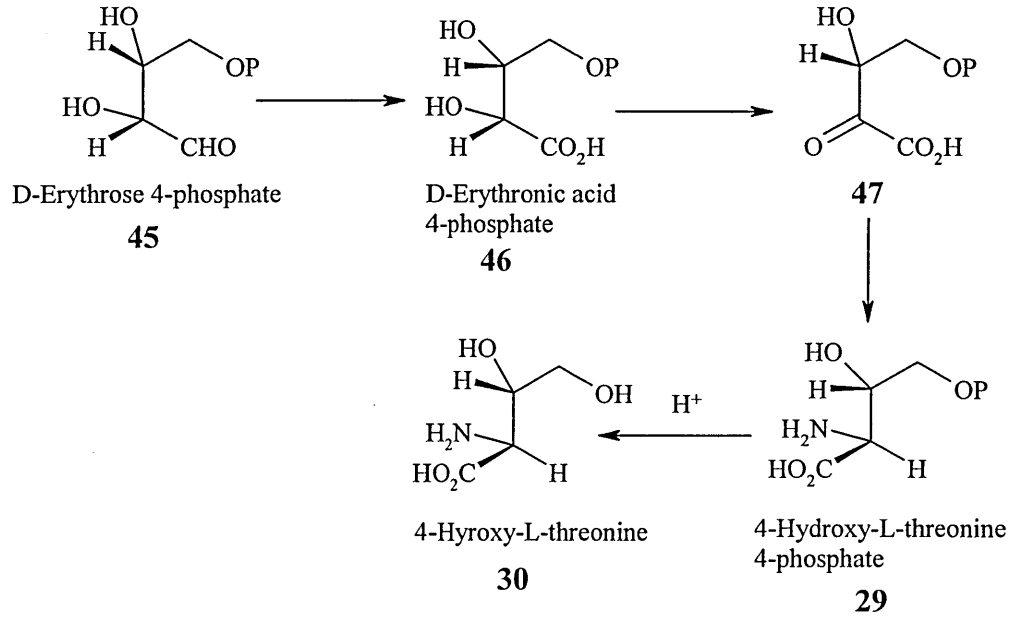


Labelled 1-aminopropane-2,3-diol

44

Scheme 13

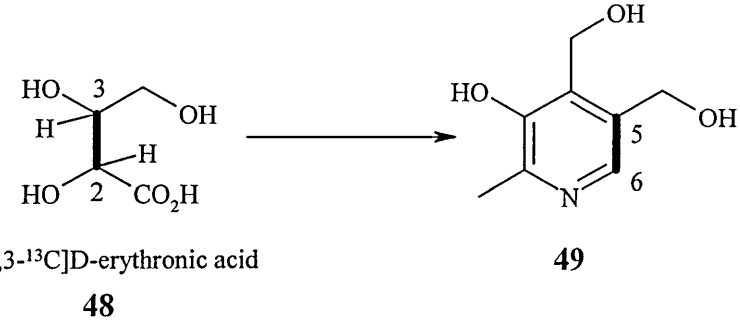
Another interpretation, based on genetic findings, suggests that the glucose-derived 3 C unit, C-6,5,5', of pyridoxine was generated not by way of a glycolytic triose phosphate intermediate, but via intermediates originating from the pentose phosphate pathway[42]. The key intermediate in the proposed formation of 4-hydroxy-L-threonine from the 4 C fragment, C-3,4,5,6, of glucose by way of the pentose phosphate pathway is D-erythrose 4-phosphate **45**, generated by a transaldolase reaction from D-sedoheptulose 7-phosphate as the 3 C donor, with D-glyceraldehyde 3-phosphate as the 3 C acceptor. D-erythrose 4-phosphate was further elaborated by oxidation at C-1 to yield the corresponding D-erythronic acid 4-phosphate **46**, which is followed by dehydrogenation at C-2 to produce α-keto acid **47**. The α-keto acid undergoes transamination to give the corresponding 4-hydroxy-L-threonine 4-phosphate **29** and phosphate ester hydrolysis yields 4-hydroxy-L-threonine **30** (scheme 14).



Biosynthesis of 4-hydroxy-L-threonine **30**

Scheme 14

Tracer evidence that supports this interpretation came from a sample of pyridoxine, isolated from an experiment with [2,3-^{13}C]D-erythronic acid **48**, which revealed that the bond-label had been transferred from the substrate into C-6,5 of pyridoxine sample **49** (scheme 15)[40].



Scheme 15

1.2.6. Genetic studies.

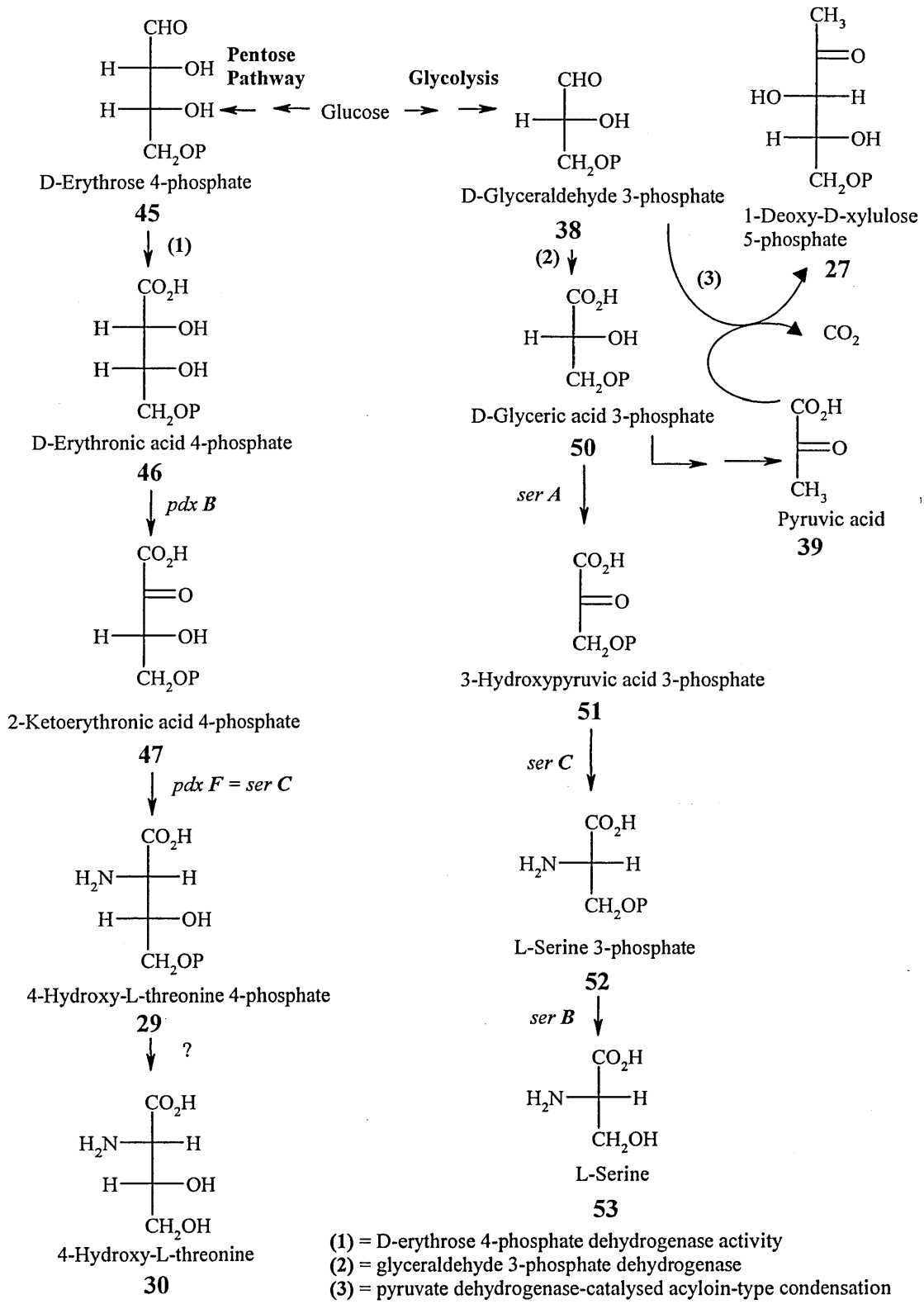
From the genetic findings, a sequence of reactions was proposed for the conversion of D-erythrose 4-phosphate **45** into 4-hydroxy-L-threonine **30** based on the similarity to the enzyme-catalysed reaction sequence from D-glyceraldehyde 3-phosphate **38** to L-serine **53** (scheme 16)[42].

In scheme 16, oxidation of D-erythrose 4-phosphate **45** to D-erythronic acid 4-phosphate **46** requires a dehydrogenase similar to glyceraldehyde 3-phosphate dehydrogenase which catalyses **38** to **50**. Evidence from crude extracts of *E. coli* mutant have shown to contain the required D-erythrose 4-phosphate dehydrogenase activity[42]. The conversion of D-erythronic acid 4-phosphate **46** into 2-ketoerythronic acid 4-phosphate **47** requires a second dehydrogenase. This step of the reaction is analogous to the transformation of D-glyceric acid 3-phosphate **50** into 3-hydroxypyruvic acid 3-phosphate **51** in serine biosynthesis. 3-Phosphoglycerate dehydrogenase, the enzyme that acts in the serine pathway, is the product of the *ser* **A** gene[57]. The *pdx* **B** gene, which encodes one of the enzymes that is required for pyridoxine biosynthesis in *E. coli*, had been isolated and sequenced[58,59]. Comparison of *pdx* **B** gene product with that of the *ser* **A** gene product, 3-phosphoglycerate dehydrogenase, revealed a similarity, and led to suggest that *pdx* **B** also encodes a 2-hydroxyacid dehydrogenase, such as the 4-phosphoerythronate dehydrogenase. 4-Phosphoerythronate dehydrogenase would be required for the production of 2-ketoerythronic acid 4-phosphate **47**. Demonstration of a synthetic sample of D-erythronic acid 4-phosphate as the substrate for *pdx* **B** gene product supports this suggestion[60]. Furthermore, when the gene product of *ser* **C**, 3-phosphoserine transaminase, was present in the incubation, the reaction proceeded only in the forward direction to the keto acid[60].

The route to 4-hydroxy-L-threonine was further supported from the fact that *pdx* **F**, another gene of the pyridoxine biosynthetic pathway[61], is in fact identical with *ser* **C**[42]. The *ser* **C** codes for 3-phosphoserine transaminase, the enzyme that catalyses the conversion of 3-hydroxypyruvic acid 3-phosphate **51** into L-serine 3-phosphate **52**. Thus, *ser* **C** plays a dual role in participating not only in the serine biosynthetic pathway but also in the pyridoxine pathway (transamination of 2-ketoerythronic acid 4-phosphate **47** to yield 4-hydroxy-L-threonine 4-phosphate **29**).

The 3-phosphoserine phosphatase encoded by *ser* **B** is the final enzyme of the serine biosynthetic pathway. The corresponding phosphatase activity that would hydrolyse 4-hydroxy-L-threonine 4-phosphate **29** to 4-hydroxy-L-threonine **30** has not been found in *E. coli*. Nor is there any evidence to equate the protein (polypeptide) products of any of the remaining identified *pdx* genes with that of *ser* **B**. It must therefore be assumed that 4-hydroxy-L-threonine 4-phosphate is the pyridoxine precursor. This implies that pyridoxine 5'-phosphate is the first vitamin to

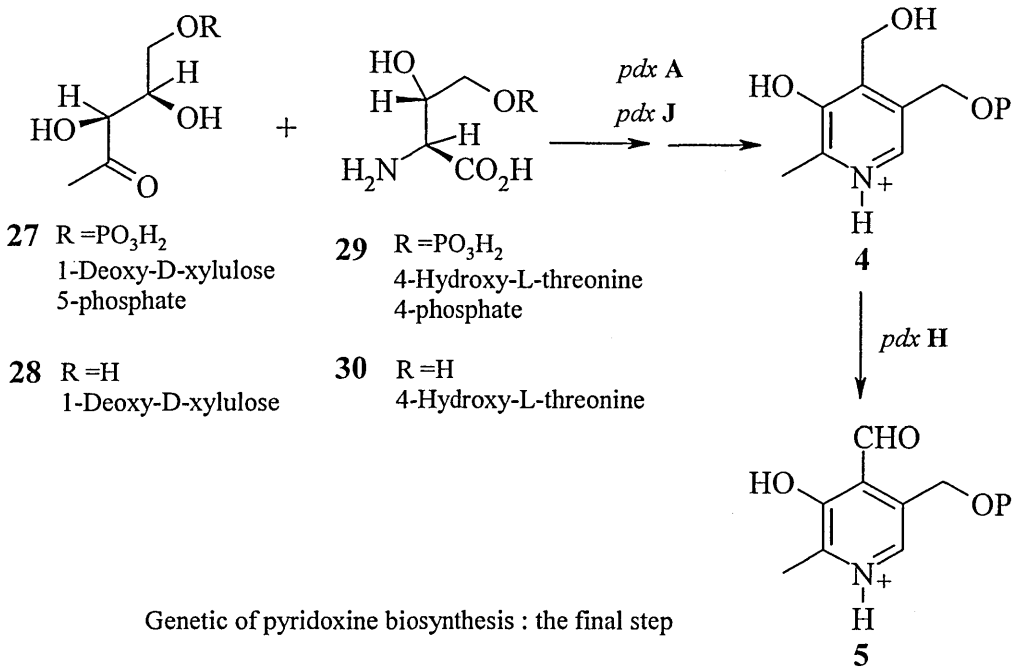
be produced or that a non-specific phosphatase, which is not the product of a *pdx* gene, is responsible for the production of the free 4-hydroxy-L-threonine **30** (scheme 16).



Analogy of the pathways to L-serine and to 4-hydroxy-L-threonine.

Scheme 16

The *pdx* **A**, *pdx* **B**, *pdx* **H**, *pdx* **J** and *ser* **C**, account for the pyridoxine biosynthetic genes that have been identified in *E. coli*. The organism uses only four dedicated enzymes, the products of *pdx* **B**, *pdx* **A**, *pdx* **J** and *ser* **C**, to elaborate the pyridoxine skeleton from primary precursors. The fifth enzyme, derived from *pdx* **H**, catalyses the conversion of pyridoxine 5'-phosphate **4** into pyridoxal 5'-phosphate **5** (scheme 17) and is not implicated in the construction of the ring system. Recent results[44,62] confirmed that the *pdx* **H** gene is the sole source of pyridoxine 5'-phosphate oxidase in *E. coli*. This enzyme is activated by flavine mononucleotide and oxygen under aerobic conditions and by FAD under anaerobic conditions.



27 R =PO_3H_2
1-Deoxy-D-xylulose 5-phosphate

28 R =H
1-Deoxy-D-xylulose

29 R =PO_3H_2
4-Hydroxy-L-threonine 4-phosphate

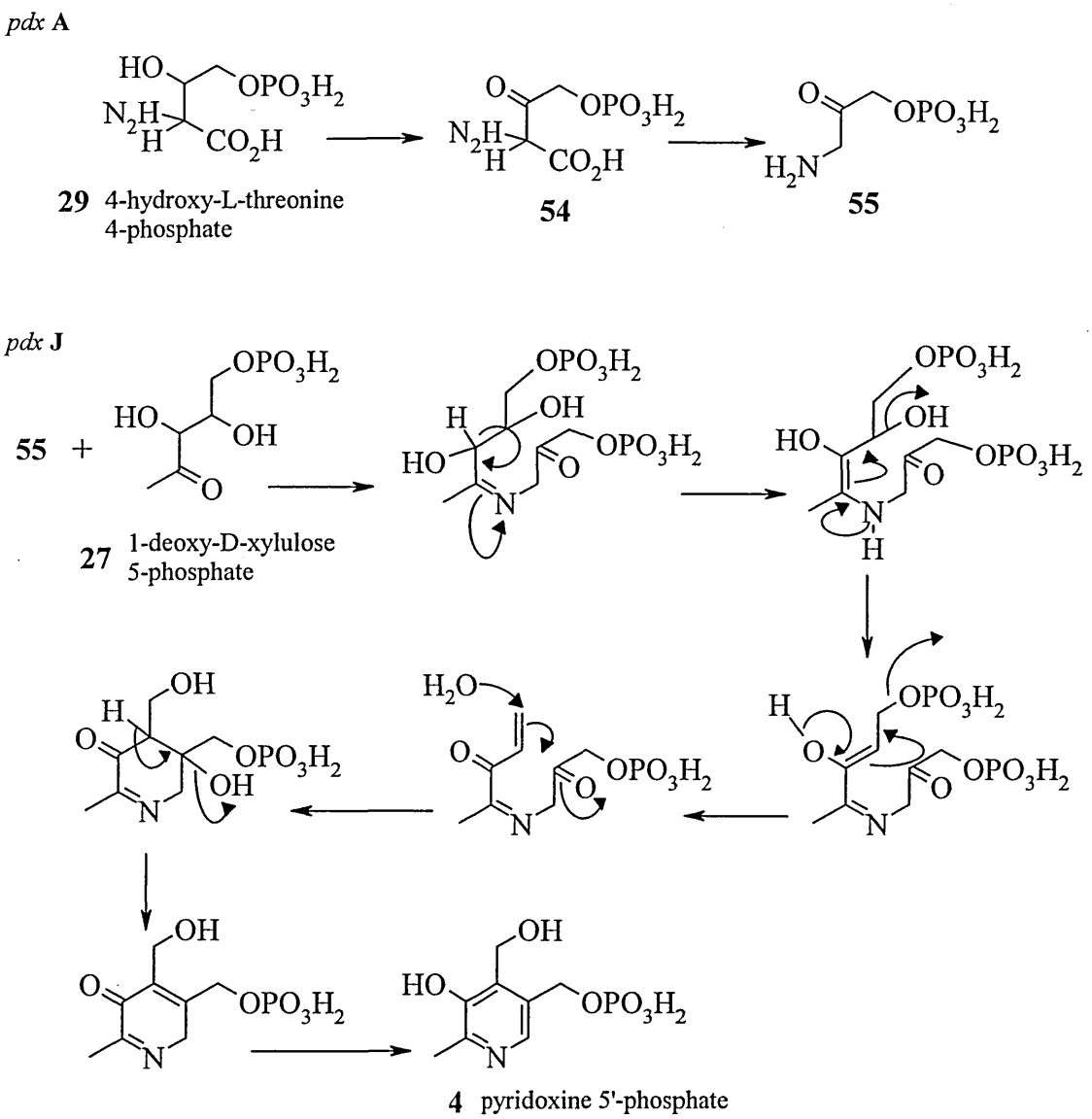
30 R =H
4-Hydroxy-L-threonine

Genetic of pyridoxine biosynthesis : the final step

Scheme 17

There is a lack of evidence for the role of the proteins that are derived from *pdx* **A** and *pdx* **J**. The fact that glycolaldehyde does not support the growth of mutants with *pdx* **A** gene suggests that the *pdx* **A** gene product catalyses a step in the pyridoxine biosynthetic pathway which is not concerned with the formation of 4-hydroxy-L-threonine[56]. Thus, *pdx* **A** gene product may be implicated in the reaction sequence that generates pyridoxine from the intermediates, 4-hydroxy-L-threonine and 1-deoxy-D-xylulose. Similarly, *pdx* **J** gene product may be assigned to the formation of the ring skeleton of the vitamin. Recent findings demonstrated that *pdx* **A** is an NAD-dependent dehydrogenase which oxidises 4-hydroxy-L-threonine 4-phosphate **29** to 2-amino-3-oxo-4-(phosphohydroxy)butyric acid **54**, followed by the

assumption that it generates 1-amino-3-(phosphohydroxy)propan-2-one **55** by spontaneous decarboxylation[63]. Furthermore, the incubation of *pdx* **J** with *pdx* **A**, 4-hydroxy-L-threonine 4-phosphate, NAD, and 1-deoxy-D-xylulose 5-phosphate demonstrated the formation of pyridoxine 5'-phosphate[64]. A reaction mechanism was proposed for the final steps in pyridoxine biosynthesis (scheme 18), and implied that the first vitamin B_6 synthesised is pyridoxine 5'-phosphate not pyridoxine.

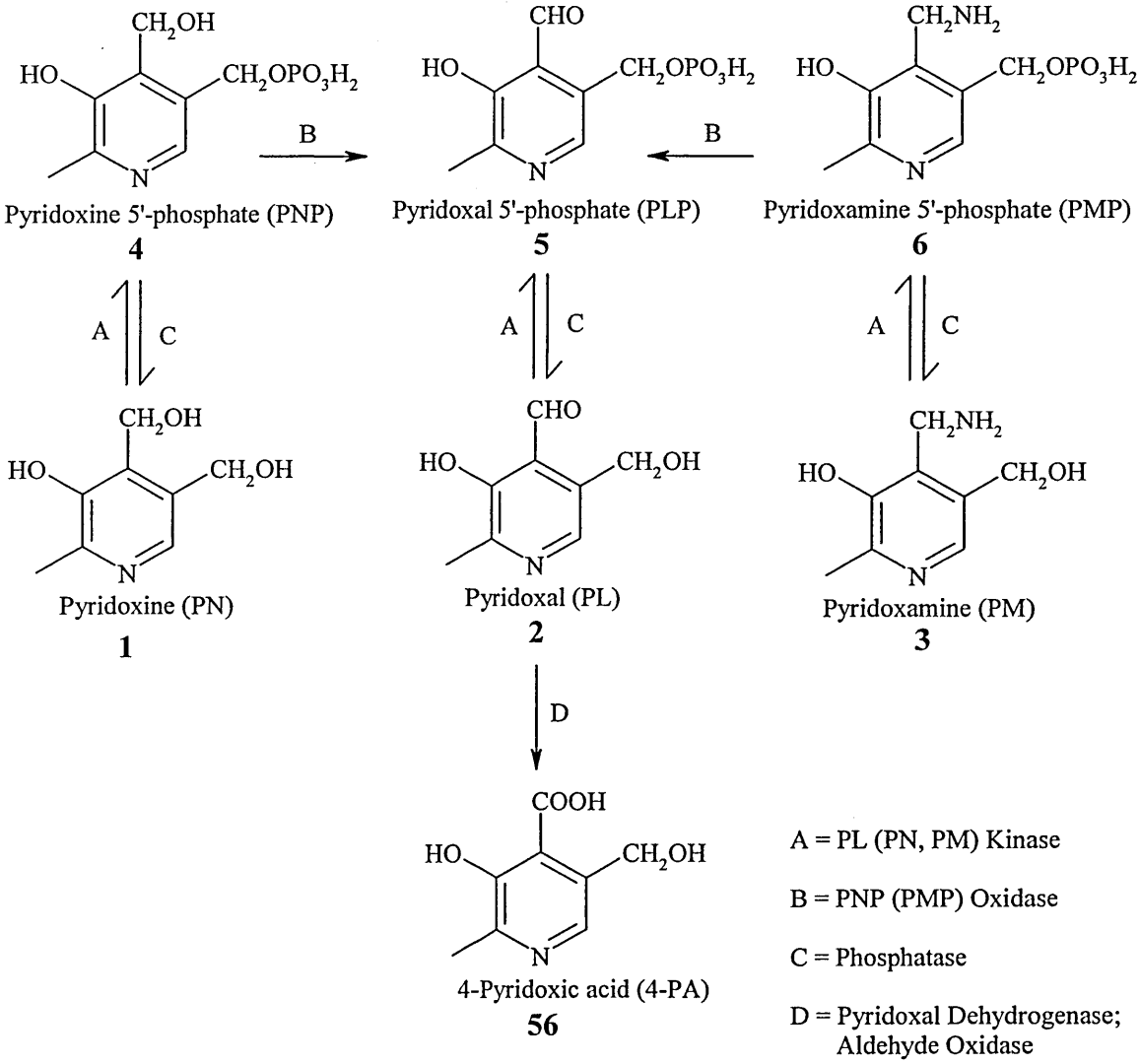


Reaction mechanism for the final steps in pyridoxine 5'-phosphate biosynthesis.

Scheme 18

1.3. Catabolic pathways of vitamin B_6.

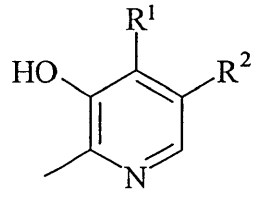
By the 1960s it was clear that the three, natural, free forms of vitamin B_6, pyridoxine **1**, pyridoxal **2**, and pyridoxamine **3**, could be transformed to the principal operating coenzyme pyridoxal 5'-phosphate **5**. The inter-conversion of various forms of vitamin B_6 are due to the actions of two types of enzymes. The first type is a kinase that catalyses phosphorylation of the 5-hydroxymethyl group of all three vitamers. The second type is an oxidase that catalyses oxidation of pyridoxine 5'-phosphate **4** and pyridoxamine 5'-phosphate **6**. Additionally recognised were the phosphatases that catalyse hydrolytic reversions of the vitaminic phosphates to restore the free vitamers. The interactions involved are shown in scheme 19.



The catabolism of pyridoxal 5-phosphate.
Scheme 19

1.3.1. Role of pyridoxal kinase.

Pyridoxal kinase catalyses the phosphorylation of all three forms of vitamin B_6 (**1**, **2**, and **3**) and appears to be present in all mammalian tissues. The kinases purified from liver, brain, and erythrocytes differ from each other in pH optima, metal requirements, and molecular weights. Pyridoxal kinase is not inhibited by pyridoxal 5'-phosphate **5** *in vitro*[65]. However, the pyridoxal 5'-phosphate content of an organ may be regulated by the rate of phosphorylation of free pyridoxal **2** by pyridoxal kinase. It was revealed in a study that daily administration of 4'-deoxypyridoxine (**7**, $R^1 = CH_3$; $R^2 = CH_2OH$) decreased the concentration of pyridoxal 5'-phosphate and increased the activity of pyridoxal kinase in rabbit brain[66].

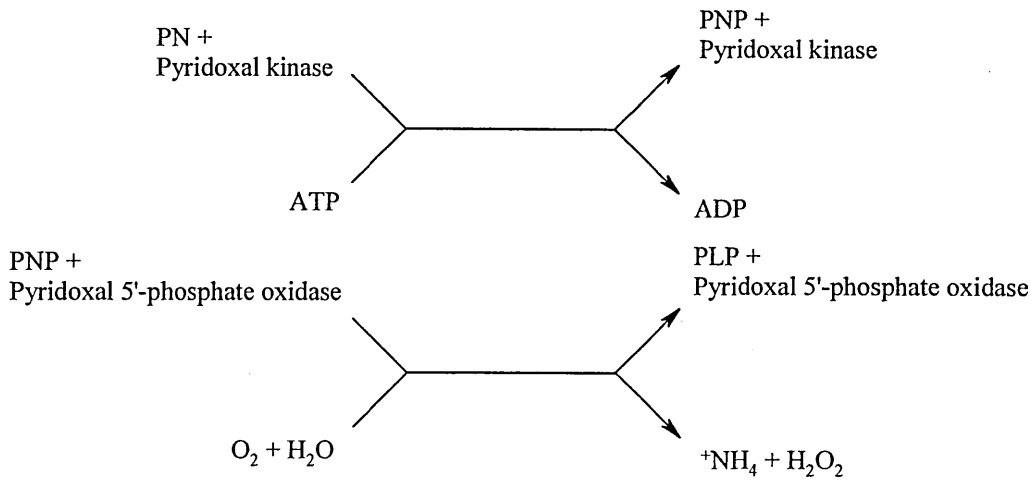


7

The results indicated that the tissue availability of pyridoxal 5'-phosphate regulated the activity of pyridoxal kinase. In a study examining the activities of pyridoxal kinase, pyridoxine 5'-phosphate phosphatase, and pyridoxine 5'-phosphate oxidase in the brains and livers of the controlled and vitamin B_6-deficient rats' revealed that only pyridoxal kinase responded rapidly to vitamin B_6 deficiency[67]. Therefore, tissue-specific responses of pyridoxal kinase might serve to protect the pyridoxal 5'-phosphate content of brain during periods of B_6 deprivation.

1.3.2. Role of pyridoxine 5'-phosphate oxidase.

Pyridoxine 5'-phosphate oxidase is an FMN-dependent enzyme that catalyses the oxidation of derivatives **4** and **6** to pyridoxal 5'-phosphate **5**. Pyridoxine 5'-phosphate oxidase, the second enzyme involved in the synthesis of pyridoxal 5'-phosphate **5**, unlike pyridoxal kinase, is inhibited by pyridoxal 5'-phosphate[68], as confirmed for crude liver oxidase[69,70] and purified pig brain oxidase[71]. Hence, some regulatory mechanisms must exist to prevent further formation of pyridoxal 5'-phosphate. In a series of kinetic and spectroscopic studies, pyridoxal kinase and pyridoxine 5'-phosphate oxidase was shown to form a complex[71] according to the reaction in scheme 20.



The formation of pyridoxal 5'-phosphate by coupling of pyridoxal kinase and pyridoxaine 5'-phosphate oxidase.

Scheme 20

Several reports[69,70,71] have confirmed that pyridoxal 5'-phosphate inhibits the activity of pyridoxine 5'-phosphate oxidase, which is a flavine mononucleotide (FMN)–requiring enzyme[72]. Interestingly, pyridoxal 5'-phosphate formation was considerably impaired in the liver of riboflavin-deficient rats[73]. Therefore, pyridoxine (pyridoxamine) 5'-phosphate oxidase could play a kinetic role[68] in regulating the level of pyridoxal 5'-phosphate formation in liver[69,70] and brain[71]. However, the control of pyridoxal 5'-phosphate formation at the level of pyridoxine (pyridoxamine) 5'-phosphate oxidase would only be effective when pyridoxine or pyridoxamine was the substrate[68].

1.3.3. Role of phosphatase.

It has been postulated[74,75] that the hydrolysis of pyridoxal 5'-phosphate **5** by pyridoxal phosphatase plays a crucial role in the regulation of the tissue content of pyridoxal 5'-phosphate. Furthermore, there is evidence[76] that newly synthesised pyridoxal 5'-phosphate is not freely exchangeable with endogenous pyridoxal 5'-phosphate but is preferentially released, converted to pyridoxal **2** by phosphatase, and then oxidised to 4-pyridoxic acid **56**.

The inhibition of phosphatase dramatically increases the concentration of pyridoxal 5'-phosphate[77]. The low plasma concentration of pyridoxal 5'-phosphate in patients with liver disease is thought to result from enhanced degradation of pyridoxal 5'-phosphate by the liver[78]. Also, children with Down's syndrome have a greater

tendency to be vitamin B_6 deficient, a deficiency which may result from a greater than normal degradation of pyridoxal 5'-phosphate[79] (table 1).

Physiological or Pathological States	**Status of Vitamin B_6**
Alcoholics	Higher degradation of PLP
Anaemia	Elevated PL-kinase
Down's syndrome	Greater susceptibility to B_6 deficiency
Leukemia	Reduced PLP in leukocytes
Peptic ulcer	Lower serum PL
Physical stress in man	Enhanced excretion of 4-PA
Physical stress in rats	Exhanced excretion of 4-PA
Pregnancy	Tendency to develop B_6 deficiency
Speed running	Elevated plasma PLP

PL = Pyridoxal, PLP = Pyridoxal 5'-phsophate, 4-PA = 4-Pyridoxic acid,

PL-kinase = Pyridoxal phosphokinase.

Table 1

1.3.4. Absorption and transport of vitamin B_6.

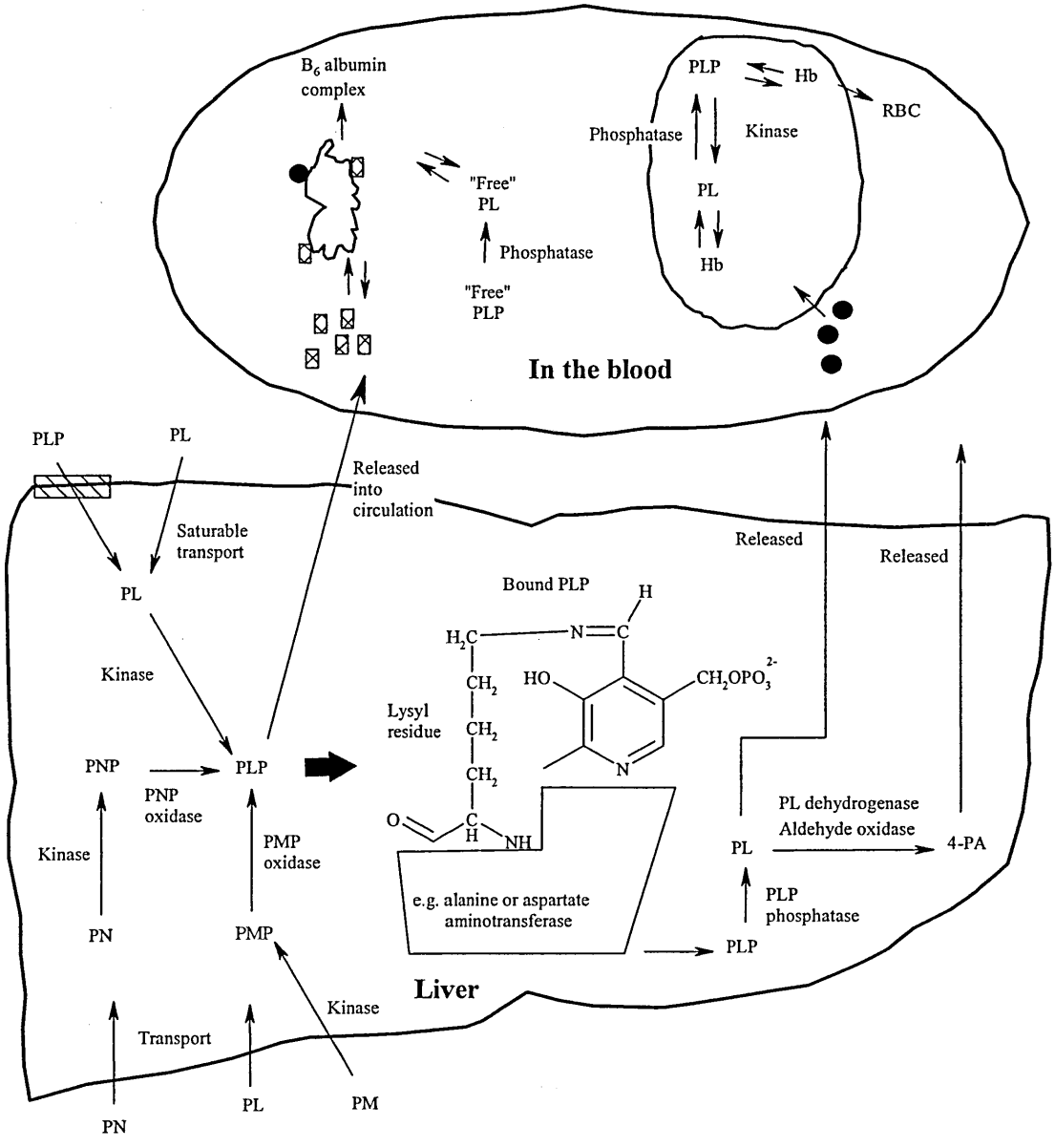
A series of studies carried out *in vivo* and *in vitro* have provided evidence[80,81,82] that:

1) Pyridoxine, pyridoxal, and pyridoxamine have comparable affinity for transport to the CNS (central nervous system).
2) The entry of ^{3}H-pyridoxine into the brain was predominantly by a transport or enzymatic phosphorylation reaction.
3) The transport of ^{3}H-pyridoxine was inhibited by non-phosphorylated B_6 derivatives.
4) Addition of unlabelled pyridoxine increased the percentage of ^{3}H-B_6 vitamers.
5) The non-phosphorylated B_6 derivatives were not retained unless phosphorylated.

These results were interpreted to indicate that the regulation of pyridoxal 5'-phosphate in the CNS depends on the transport of non-phosphorylated derivatives and their subsequent intracellular phosphorylation by pyridoxal kinase. Since the non-phosphorylated derivatives are not retained, the significance of pyridoxal kinase becomes apparent. However, studies have shown that pyridoxal 5'-phosphate enters erythroid precursor cells without prior dephosphorylation[83]. Similarly, studies on the intestinal disappearance of pyridoxal 5'-phosphate in rats seem to indicate that although a major portion of pyridoxal 5'-phosphate is dephosphorylated (catalysed by intestinal phosphatases) and then transported as pyridoxal, a second mechanism also exists by which pyridoxal 5'-phosphate is transported unchanged[84,85]. The transport of [^{14}C]pyridoxal 5'-phosphate and [^{14}C]pyridoxine into isolated rat mitochondria indicates that pyridoxal 5'-phosphate can rapidly enter the intermembrane space of isolated mitochondria, but its penetration into the matrix occurs at a slower and more sustained rate[66]. Hence, the transport of pyridoxal 5'-phosphate into isolated rat liver mitochondria is energy dependent, taking place by passive diffusion facilitated by protein binding[66].

1.3.5. Role of protein binding.

Protein binding of pyridoxal 5'-phosphate, which has been postulated to be tissue specific, may regulate the steady-state concentration of pyridoxal 5'-phosphate. Studies have shown that in rat liver, 66 % of pyridoxal 5'-phosphate is localised in the cytosolic fraction and this pool of coenzyme is preferentially depleted under conditions of vitamin B_6 deficiency[86]. Furthermore, up to 88 % of cytosolic pyridoxal 5'-phosphate is bound to proteins with molecular weights of 1.2 x 10^5 daltons. Among five proteins that show high-affinity binding for pyridoxal 5'-phosphate, three were pyridoxal 5'-phosphate-dependent enzymes, alanine aminotransferase, aspartate aminotransferase, and glycogen phosphorylase (scheme 21).



PL = pyridoxal
PLP = pyridoxal 5'-phosphate
PN = pyridoxine
PNP = pyridoxine 5'-phosphate

PM = pyridoxamine
PMP = pyridoxamine 5'-phosphate
4-PA = 4-pyridoxic acid
Hb = haemoglobin
RBC = red blood cell

Simplified representation of the transport of pyridoxal into liver
and conversion into pyridoxal 5'-phophate, and the release of pyridoxal into circulation.

Scheme 21

The storage and regulation of pyridoxal 5'-phosphate in the liver and muscle are different. In the muscle, glycogen phosphorylase stores up to 90 % of the pyridoxal 5'-phosphate, whereas in the liver, this binding accounts for only 10 % of the bound pyridoxal 5'-phosphate[86]. Studies in human red blood cells have shown that pyridoxal

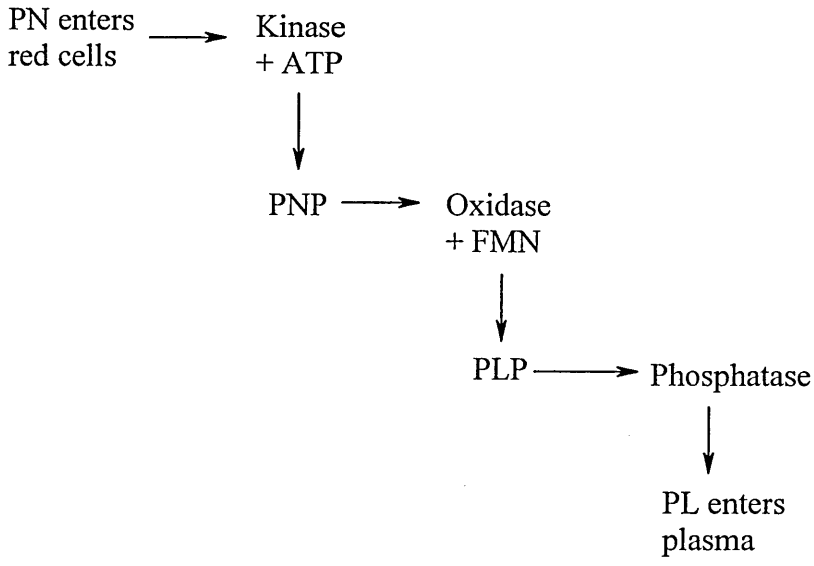
and pyridoxal 5'-phosphate also binds to haemoglobin[87]. In general, the protein binding of vitamin B_6 is thought to accomplish the following objectives:[76,86,87,88]

1) The binding of pyridoxal 5'-phosphate to proteins prevents its entry into the cells.
2) The bound pyridoxal 5'-phosphate is not hydrolysable.
3) The bound pyridoxal 5'-phosphate is physiologically inactive.
4) The bound pyridoxal 5'-phosphate is not available to pyridoxal 5'-phosphate-dependent enzymes.
5) The bound pyridoxal is not phosphorylated.

1.3.6. Pyridoxal as a major source of circulating vitamin B_6.

Pyridoxal, the product of phosphatase-mediated catabolism, has assumed a greater role in vitamin B_6 metabolism than was once believed. The transport and accumulation of pyridoxine and pyridoxal by erythrocytes have been studied using the rapid-mixing techinques with ^{3}H-labelled substrates[89]. These studies have shown that erythrocytes transport pyridoxine and pyridoxal by passive diffusion. Furthermore, the initial influx of pyridoxine or pyridoxal is not saturated and is not affected by pyridoxamine or 4'-deoxypyridoxine. The accumulation of [^{3}H]pyridoxine against a concentration gradient was due to phosphorylation of pyridoxine to pyridoxine 5'-phosphate, and the pyridoxal was accumulated by a kinase-independent mechanism. Gel filtration studies indicate that pyridoxal can accumulate by binding to an intracellular protein, most probably haemoglobin[89] (see scheme 21).

Human erythrocyte have shown to take up [^{3}H]pyridoxine, with at least 99 % of the radioactivity appearing in the supernatant fraction[90]. Eighty percent of that radioactivity was in pyridoxal 5'-phosphate and was bound. The newly synthesised pyridoxal 5'-phosphate was bound to haemoglobin[90]. Hence, pyridoxine is metabolised in human red cells[91] according to scheme 22. However, the erythrocytes of rats and some other species exhibits no oxidase activity[89,92] and the pyridoxine 5'-phosphate has no known fate other than hydrolysis back to pyridoxine, which finds its way to the plasma and then to other tissues for conversion to pyridoxal 5'-phosphate.

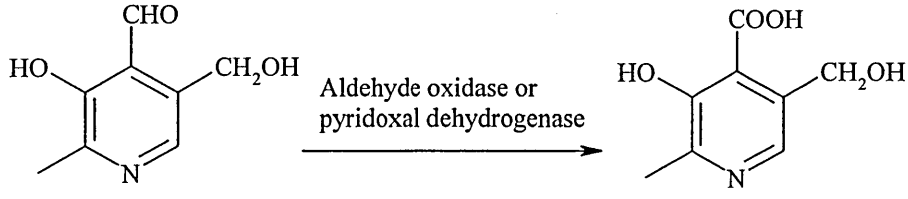


Scheme 22

Studies with human platelets revealed that pyridoxal kinase has greater affinity for pyridoxal and pyridoxamine than for pyridoxine[93]. Therefore, pyridoxal kinase may play a role in regulating the synthesis of pyridoxal 5'-phosphate in erythrocytes[94]. The transport and metabolism of [^{3}H]pyridoxal and [^{3}H]pyridoxal 5'-phosphate in the small intestine of rats revealed that pyridoxal 5'-phosphate, after hydrolysis in the lumen, is transported chiefly as pyridoxal[85]. Since pyridoxine is not bound to any plasma proteins[95], it could not serve as a storage form of vitamin B_6, therefore, the mammalian system tends to conserve pyridoxal. In the kidney, pyridoxine at high concentration is secreted from the kidney tubules by a saturable process whereas pyridoxal, in either physiological or pharmacological doses, does not undergo tubular secretion[96]. Liver and intestine are very active in their inter-conversion of B_6 vitamers. When pyridoxine is taken up by these cells, it is rapidly acted on by pyridoxal kinase and then converted to pyridoxal 5'-phosphate by pyridoxine 5'-phosphate oxidase[92]. These two enzymes plus phosphatase provide a means of converting dietary pyridoxine to circulating pyridoxal, which can then serve as a source of the coenzyme pyridoxal 5'-phosphate in all tissues that contain pyridoxal kinase, whether they contain pyridoxine 5'-phosphate oxidase or not.

1.3.7. Catabolism of pyridoxal to 4-pyridoxic acid in mammalian tissues.

4-Pyridoxic acid **56**, the major vitamin B_6 excretory product, is formed from pyridoxal **2** by the action of aldehyde oxidase[97] or by the action of an NAD-dependent aldehyde dehydrogenase[98] (scheme 23). While pyridoxal is acted on by dehydrogenase from most tissues[70], pyridoxal is a substrate for the aldehyde oxidase only in the liver[99]. Dehydrogenase activity is found in the mitochondria, cytosol, and microsomes in many tissues, and these enzymes have low substrate specificity. Under physiological conditions, it appears that the dehydrogenase is more involved in forming 4-pyridoxic acid than is the oxidase.



2 Pyridoxal (PL) **56** 4-Pyridoxic acid (4-PA)

Scheme 23

1.3.8. Role and properties of aldehyde oxidase.

One physiological role of aldehyde oxidase is to catabolise the oxidation of N^1-methylnicotinamide. The second role of aldehyde oxidase is to oxidise pyridoxal to 4-pyridoxic acid[100,101].

Aldehyde oxidase (aldehyde-oxygen oxidoreductase) readily oxidises large numbers of unsubstituted and carbon monosubstituted heterocycles and has preference for compounds with a substituted pyridine ring[97,99]. It is an exclusively cytoplasmic enzyme which contains non-heme iron, molybdenum, and flavine adenine dinucleotide in the ratios of 4:1:1, along with a coenzyme Q-like quinone. In addition to molecular oxygen, the internal electron transport chain is capable of functioning with a variety of electron receptors[102].

Aldehyde oxidase is capable of oxidising pyridoxal to 4-pyridoxic acid, but pyridoxal 5'-phosphate does not serve as a substrate[97,103]. N^1-Methylnicotinamide is able to compete with pyridoxal for oxidation *in vivo*[100]. Inactivation of N^1-methylnicotinamide oxidase results also in an equal loss of oxidase activity toward pyridoxal. A study with mutant rats endowed with high, low, or no aldehyde oxidase

activity, revealed that when challenged with pyridoxine, animals with no aldehyde oxidase activity excreted as much 4-pyridoxic acid as animals with low or high enzyme activity[100]. Therefore, an alternate pathway must be operational in the catabolism of pyridoxal. Furthermore, although aldehyde oxidase is capable of catabolising pyridoxal as a substrate, this pathway is of dubious significance and plays a negligible role in physiological catabolism of pyridoxal[101].

1.3.9. Role and properties of pyridoxal dehydrogenase (aldehyde dehydrogenase).

The NAD^+-dependent aldehyde dehydrogenase (aldehyde-NAD^+ oxidoreductase) is capable of catabolising pyridoxal to 4-pyridoxic acid[98]. NAD^+-Dependent aldehyde dehydrogenase with a broad affinity toward biological aldehydes has been detected in many mammalian tissues[104]. Since, mutant rats without aldehyde oxidase were able to catabolise pyridoxal, an alternate pathway must exist according to the following reaction[98] (scheme 24):

$$PL + NAD^+ + H_2O \xrightarrow{\text{Pyridoxal dehydrogenase}} \text{4-Pyridoxic acid} + NADH + H^+$$

Scheme 24

1.3.10. Summary of Catabolic Pathways.

The formation and degradation of pyridoxal 5'-phosphate, and the circulation of pyridoxal have been summarised in scheme 21. Factors such as the transport of the precursors, the protein binding of pyridoxal 5'-phosphate, the activity of pyridoxal kinase and pyridoxal 5'-phosphate phosphatase regulates the concentration of pyridoxal 5'-phosphate. In human, the catabolic pathways of vitamin B_6 are simplified in the following:

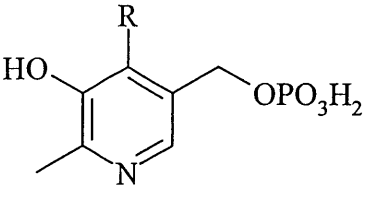
1) Phosphorylated forms of B_6 must first be dephosphorylated.
2) Absorption of pyridoxal, pyridoxine, and pyridoxamine occurs primarily in the small intestine by passive diffusion.
3) Within intestinal cell, pyridoxine and pyridoxal are converted to pyridoxine 5'-phosphate and pyridoxal 5'-phosphate, respectively. Pyridoxine 5'-phosphate may be converted to pyridoxal 5'-phosphate.

4) Pyridoxal 5'-phosphate, pyridoxal, and some pyridoxamine are bound to albumin for transport in plasma.
5) In the liver, unphosphorylated forms are phosphorylated and pyridoxine and pyridoxamine 5'-phosphate are generally converted to pyridoxal 5'-phosphate.
6) In the tissues, only pyridoxal is taken up and pyridoxal 5'-phosphate must be hydrolysed before uptake.
7) Within the cells, pyridoxal is phosphorylated by pyridoxal kinase.
8) Pyridoxine 5'-phosphate/pyridoxamine 5'-phosphate oxidase in tissues converts pyridoxine 5'-phosphate and pyridoxamine 5'-phosphate into pyridoxal 5'-phosphate, the coenzyme form of vitamin B_6.
9) Pyridoxic acid is the major excretory product in urine.

1.4. Pyridoxal 5'-phosphate binding sites in enzymes.

Many enzyme-catalysed reactions require a substance to be present in addition to the enzyme and the substrate in order for the reaction may proceed. Such substances, known as coenzymes or cofactors, form an essential part of the catalytic mechanism. The intact enzyme system, or holoenzyme, is thus formed from a protein portion called the apoenzyme and a non-protein component referred to as a prosthetic group, a cofactor, or more commonly, a coenzyme. The combination of the coenzyme, pyridoxal 5'-phosphate, with the protein apoenzyme creates a functional enzyme. It is the interplay between the coenzyme and the protein that leads specifically to the binding, hence the type of reaction being catalysed.

Pyridoxal 5'-phosphate **5** and pyridoxamine 5'-phosphate **6** are the two central coenzymes of amino acid metabolism[105]. The synthesis of almost all amino acids is achieved by the biochemical reaction of pyridoxamine 5'-phosphate with a α-keto acid in one of the steps of an overall process referred to as transamination. The pyridoxamine 5'-phosphate is converted to pyridoxal 5'-phosphate (the other coenzyme form) which can react with a second amino acid to regenerate pyridoxamine 5'-phosphate and convert the amino acid to its corresponding keto acid. As a result, a keto acid and an unrelated amino acid interchange functionality.



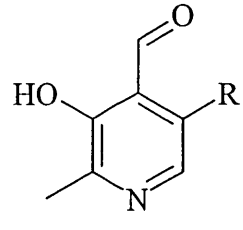
5 R = CHO
Pyridoxal 5'-phosphate

6 R = CH_2NH_2
Pyridoxal 5'-phosphate

Pyridoxal 5'-phosphate **5** has a number of other functions as well. It is the coenzyme for most of the interesting metabolic transformations of amino acids, catalysing such processes as α-decarboxylation, α,β-elimination reactions, β-substitution reactions (as in the reaction of serine with indole to form tryptophan). In the pyridoxal 5'-phosphate, certain functional groups are required specifically for binding to the apoenzyme. All pyridoxal 5'-phosphate-dependent enzymes bind the aldehyde

component of pyridoxal 5'-phosphate through the interaction with the ε-amino group of a lysine residue to form the Schiff's base. In the transaminases, there is an obligatory conversion of the aldehyde to amine form as pyridoxamine 5'-phosphate. This reaction, however, may also occur as a side reaction with other enzymes such as glutamate decarboxylase[106] and aspartate β-decarboxylase[107]. In all cases, the affinity for this form of the coenzyme is reduced and reactivation of the enzymes may be accomplished by addition of pyridoxal 5'-phosphate.

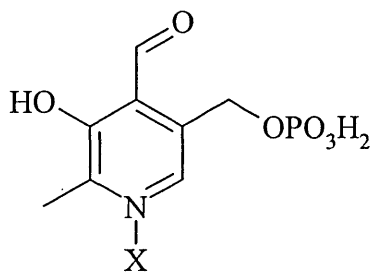
The phosphate group, along with the aldehyde moiety, shows the uniform response needed for most pyridoxal 5'-phosphate-dependent enzymes. Enzymes such as aspartate transaminase, phosphorylase, and serine dehydratase[108,109] have shown a preference for the dianionic state of the phosphate, whilst the monoionic state suffices with other enzymes and even monomethyl esters are effective as coenzyme analogues in serine transhydroxymethylase[110]. In most cases, enzymes are quite sensitive to replacements in this region of the coenzyme, and these effects are more likely to be due to steric protein requirements than specific needs of particular states of ionisation of the phosphate for catalysis. In fact, sulphate in the form of pyridoxal 5'-sulphate **57** can substitute for pyridoxal 5'-phosphate in enzymes such as arginine decarboxylase and tryptophanase[111]. The presence of the ester is not necessarily a requirement for efficient binding either and phosphonate analogues **58** also show efficient binding in serine dehydratase[111] and aspartate transaminase[112]. In some enzymes, catalysis can be mediated when the pyridoxal is used alone. The removal of the phosphate ester, however, greatly reduces the efficiency of the catalytic events in enzymes such as the aspartate transaminase isozymes[113] and lends support for the need of phosphate as an anchor to bind pyridoxal 5'-phosphate in the proper catalytic arrangement of enzyme-active sites[17].



57 R = CH_2OSO_3H

58 R = $CH_2OPO(OH)CH_3$

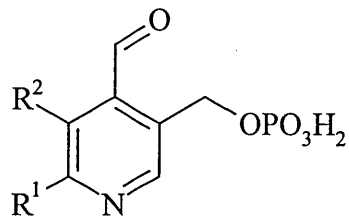
In aspartate transaminase, the pyridine nitrogen group appears to be required for binding in its protonated form[114]. However, pyridoxal 5'-phosphate *N*-oxide **59** and *N*-methyl pyridoxal 5'-phosphate **60** derivatives can be accepted as coenzyme analogues in some enzymes. These derivatives can even induce some coenzyme catalytic effect in aspartate transaminase[112] and regenerate as much as 24% activity in glutamate decarboxylase[115].



59 X = O

60 X = CH_3

The 2-methyl group was considered to take some part in the binding of coenzyme to apoenzyme by hydrophobic interaction. Although this bond is not strictly necessary for the enzyme-catalysed reaction to proceed, it may assist in the fine adjustment of the spatial interrelations between coenzyme and substrate[116]. The analogue, 2-norpyridoxal 5'-phosphate **61**, which lacks a methyl substitutent, was a slightly better catalyst for transamination in aspartate transaminase[117]. In other enzymes, 2-norpyridoxal 5'-phosphate varies, as it is a better aid for catalysis for arginine decarboxylase but less active than pyridoxal 5'-phosphate for tryptophanase[118].



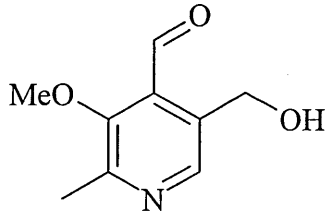
61 R^1 = H, R^2 = OH

62 R^1 = CH_3, R^2 = OCH_3

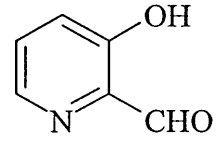
However, the phenolic group at the 3-position of the pyridine ring seems not to be necessary for enzymic binding purposes, since the 3-*O*-methyl pyridoxal 5'-phosphate **62** has been shown to bind efficiently to apoaspartate transaminase[112].

1.4.1. The role of pyridoxal 5'-phosphate in model systems.

The general mechanism for enzymic reactions involving pyridoxal 5'-phosphate was proposed based on non-enzymatic reactions between amino acids and pyridoxal[2, 119]. Early studies[120, 121] showed the significance of the various structural features of the pyridoxal molecule with respect to its ability to catalyse the transamination process in the presence of metal-ions. The derivative 3-*O*-methyl pyridoxal **63** was not effective as a catalyst, and indicated a free hydroxyl group, *ortho-* to the formyl group, as necessary. 2-Formyl-3-hydroxypyridine **64** was active as a catalyst, as its structure is electronically similar to that of pyridoxal. The methyl and the hydroxymethyl groups appear not to be important in non-enzymic reactions. In other reactions catalysed by pyridoxal, the pyridine ring may be replaced by a benzene ring carrying a nitro-group. This emphasises the role of the heterocyclic ring as an electron-attracting species.

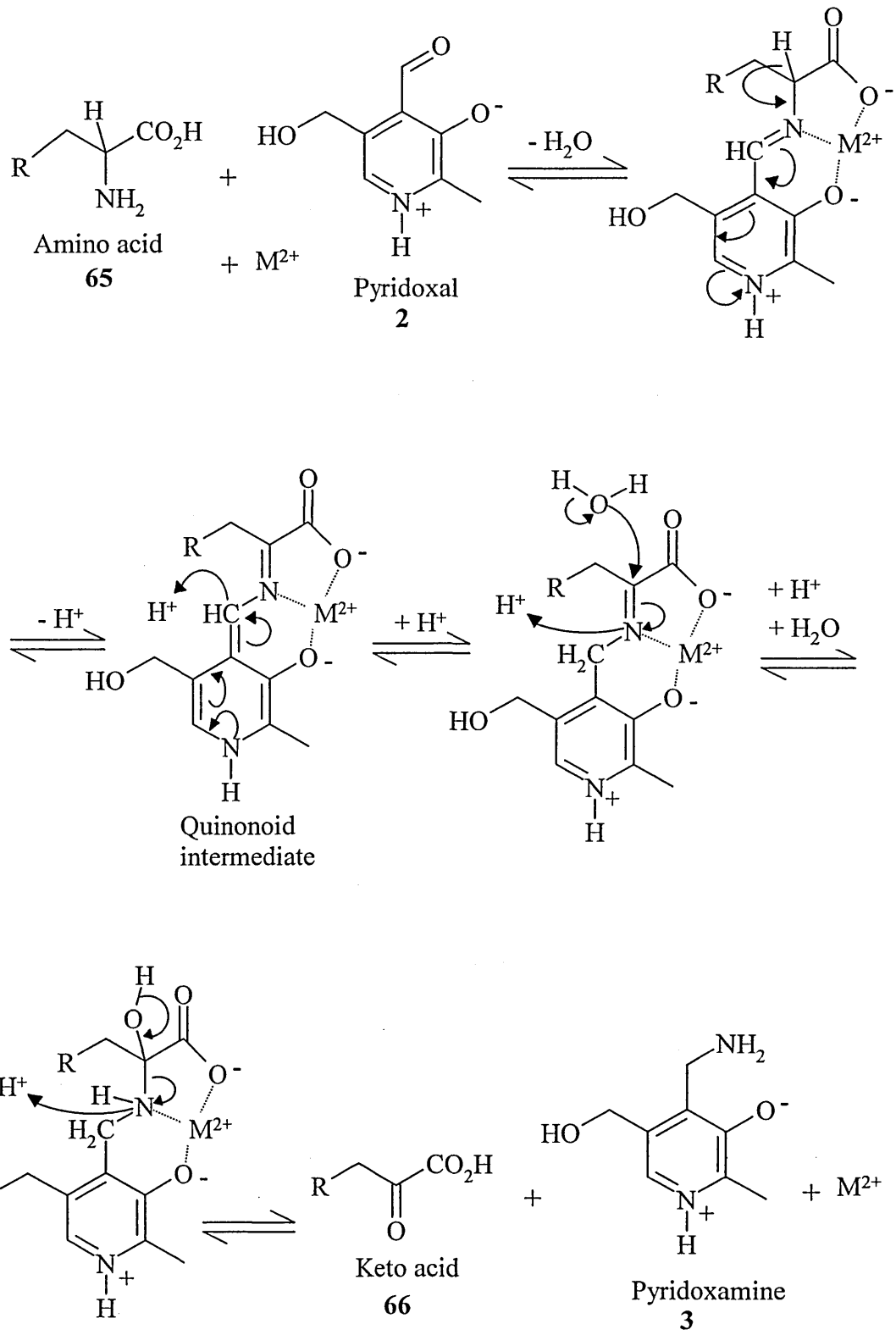


63



64

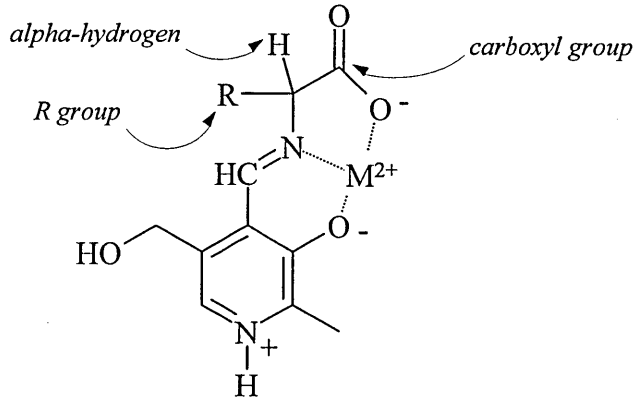
Model experiments typically involved pyridoxal **2**, a polyvalent metal ion (Ca^{2+}, Fe^{3+}, Al^{3+}), and the appropriate amino acid substrate **65**. In non-enzymic transamination reactions, the motivating force for the reaction is the stabilisation of the transition state for carbanion formation by delocalisation of the charge through the conjugated system. The function of the metal ion in these model systems appears to be stabilisation of the intermediate imine, which increases the inductive effect on the α-carbon atom, thus facilitating release of the proton (scheme 25). The model experiments suggest that the essential feature of pyridoxal 5'-phosphate mediated reactions is the formation of an imine (Schiff's base) between the α-amino group of the amino acid and the aldehyde group of pyridoxal 5'-phosphate[122].



Scheme 25

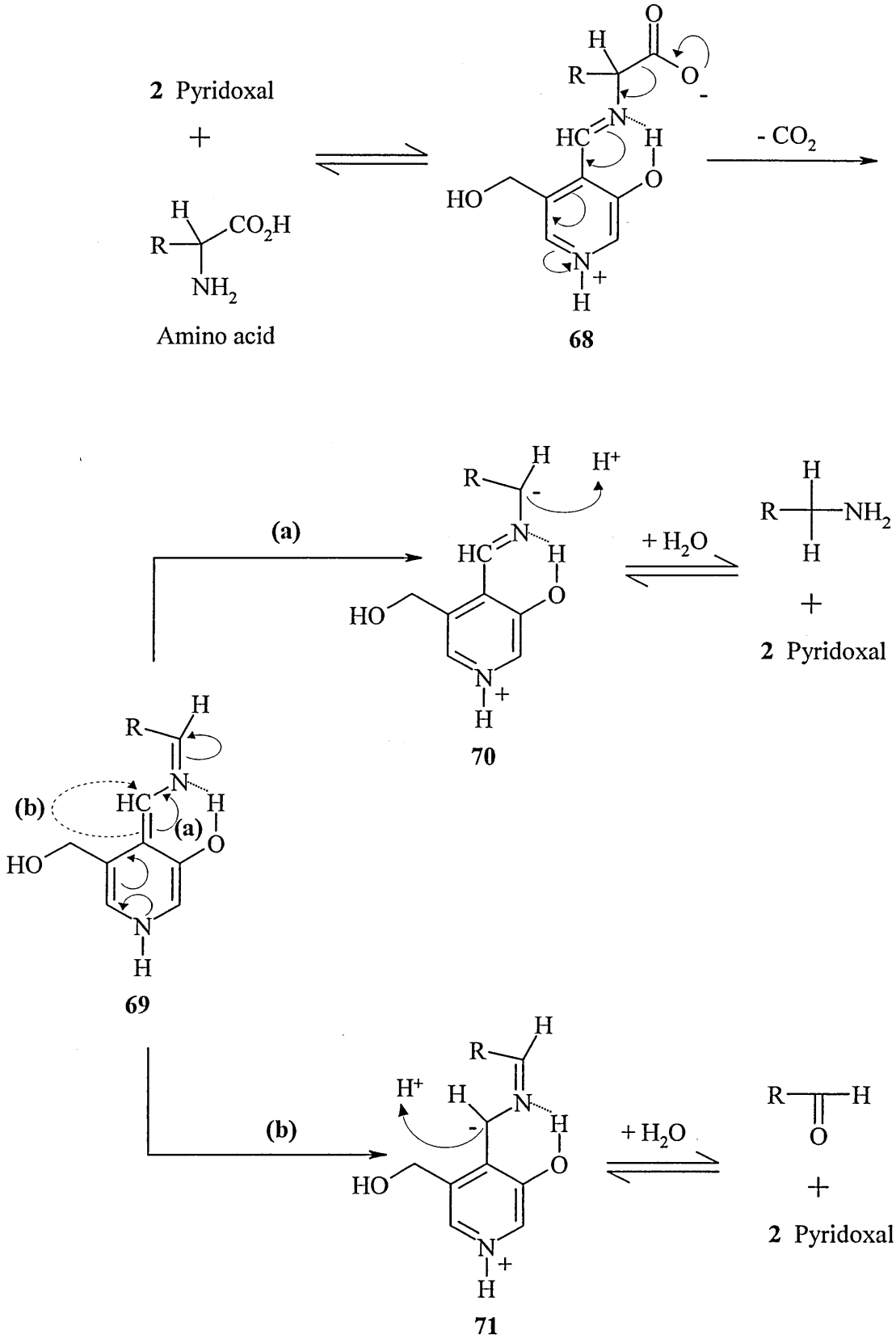
Using model systems similar to non-enzymic transaminations, pyridoxal is able to non-enzymically catalyse decarboxylation, racemisation, elimination, and condensation reactions[123]. The proposed mechanisms all involve the prior formation

of pyridoxal-amino acid Schiff's bases as in the case of the transamination reactions. In intermediate **67**, the presence of the protonated pyridine ring activates or labilises the carboxyl group, the α-hydrogen or the R group of the amino acid species.



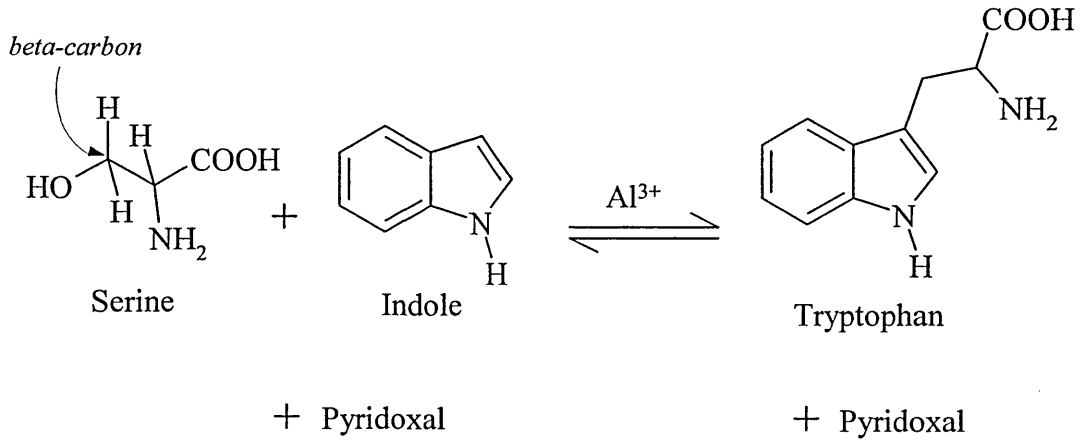
67

In the process of decarboxylation, the α-amino acid undergoes elimination of carbon dioxide and the molecule takes up a proton from the solvent. The α-amino acid reacts with pyridoxal **2** to give **68**, which loses carbon dioxide to produce intermediate **69**. The transitional form **69** may stabilise as **70**, which hydrolyses to the corresponding amine and pyridoxal, or as **71**, which hydrolyses to an aldehyde or ketone and pyridoxamine (scheme 26). The latter decarboxylation-dependent transamination reaction has no known enzymatic counterpart. The decarboxylation process differs from most of the other pyridoxal-catlysed reactions in that the α-hydrogen of the amino acid species remains attached to the carbon throughout. The absence of any necessary reaction in which this α-hydrogen is lost, is shown by the observation that amino acids carrying substituents other than hydrogen undergo decarboxylation readily[124]. However, the presence of metal ions actually inhibit the decarboxylation of α-amino acids in these model systems, since the carboxyl group forms part of the chelate structure where the pair of electrons on the carboxyl anion is shared with the metal ion. These features would be expected to decrease the tendency for carbon dioxide to be eliminated, as the metal chelates would not be able to attain the preferred conformation for decarboxylation, It is most probable then that, even when metal-ions are present, decarboxylation occurs in the metal-free complex[124, 125].


2 Pyridoxal
+
Amino acid
68
- CO_2
(a)
(b)
70
69
71
+ H_2O
+ H_2O
2 Pyridoxal
2 Pyridoxal

Scheme 26

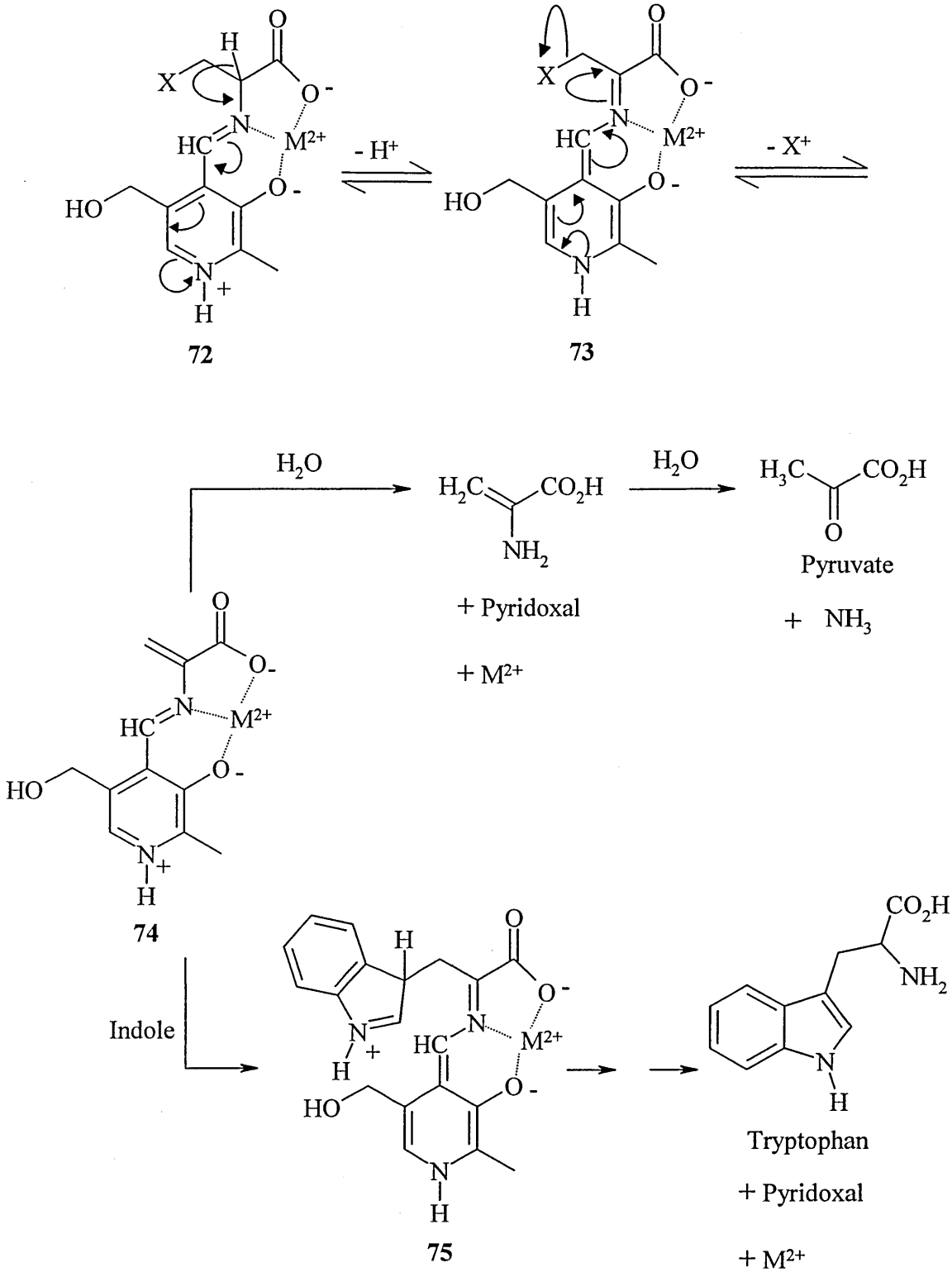
In non-enzymic model systems, the nature of the substrate and the experimental conditions determine which type of reaction will occur. The use of an amino acid such as serine, with a leaving group in the β-position, in the presence of indole as nucleophile will lead to the non-enzymic transformation of serine into trytophan (scheme 27).



Scheme 27

The pH of the reaction environment controls whether the proton returns to the α-carbon position (racemisation) or to the aldehyde carbon atom (transamination). The transamination reaction is favoured at lower pH values (*ca.* pH 5) and racemisation at higher pH values (*ca.* pH 10). Racemisation is the reversal of the reaction from **73** to **72** in scheme 28, where the de-protonated α-carbon is non-stereospecifically re-protonated. The intermediate imine **73** can undergo elimination reaction, leading to the intermediate **74**. Hydrolysis of this intermediate leads to pyruvate and ammonia. Alternatively, reaction between the intermediate and indole will lead to intermediate **75** and hence to tryptophan.

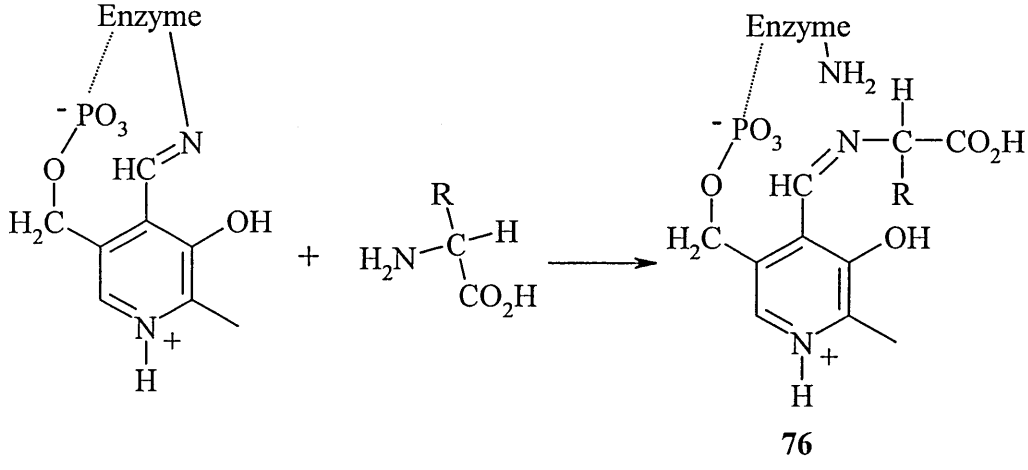
The model systems used pyridoxal plus metal ions in duplicating many enzymic reactions of α-amino acids and tend to suggest that metal ions might play an important part in the corresponding enzymic reactions. However, highly purified enzyme systems have been prepared which require pyridoxal 5'-phosphate and do not require metal ions for full activity[125]. In the model systems, the function of the metal ion is to maintain the correct geometry of the intermediate imines and thus facilitate charge delocalisation.



Scheme 28

In the enzymic reaction, the enzyme protein maintains the geometry. Thus, the role of pyridoxal 5'-phosphate in the enzymic reaction is very similar to that of pyridoxal in the model system. Since the initial reaction between coenzyme and apoenzyme is the

formation of an imine between pyridoxal 5'-phosphate and the ε-amino group of lysine to give the holoenzyme, a transamination reaction must take place in the presence of the amino acid substrate to give the imine **76** involved in the enzymic reaction (scheme 29).



76

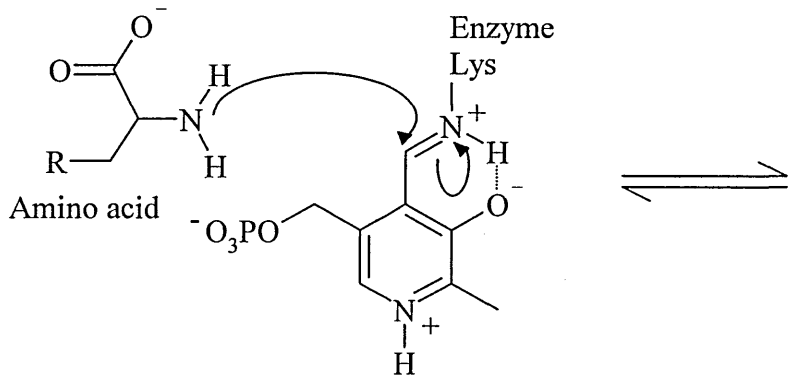
Scheme 29

1.4.2. Pyridoxal 5'-phosphate in enzymic reactions.

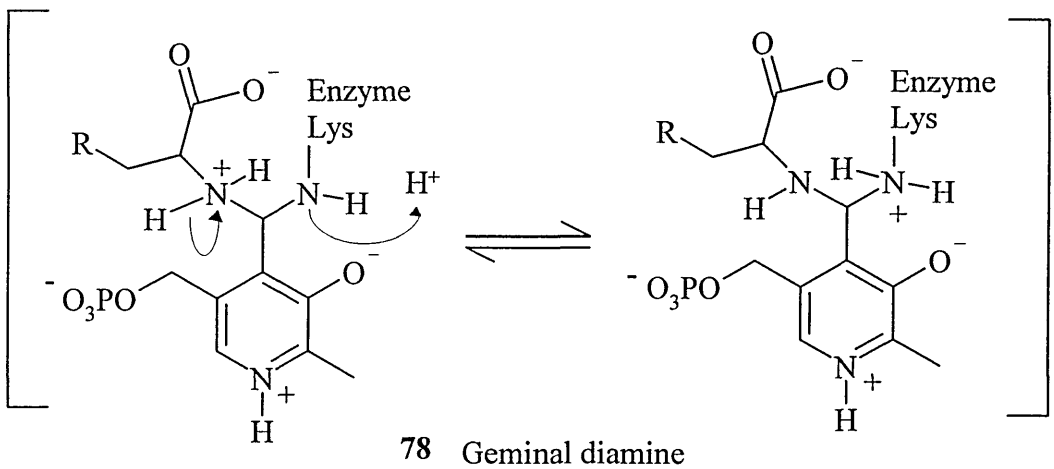
The coenzyme, pyridoxal 5'-phosphate, has two basic chemical properties; through its aldehyde group, it forms an imine with the amino group of substrate and, because it acts as an 'electron sink', it withdraws electrons from the substrate. In the absence of apoenzyme, numerous different reactions would occur simultaneously, but the unique environment provided by the protein part of each different pyridoxal 5'-phosphate-dependent enzyme directs the basic catalytic properties of the coenzyme to provide the holoenzyme with its own substrate and reaction specificity.

The early steps in all reactions catalysed by pyridoxal 5'-phosphate-dependent enzymes on amino acids are essentially the same. The coenzyme is always bound as an imine to the ε-amino group of a lysine residue in a structure known as the 'internal aldimine' **77**. After initial binding as a Schiff's base, the amino group of the lysine residue is exchanged for the amino group of the substrate amino acid to form the external aldimine **79**, the process is also known as transaldimination. Transamination itself is not a single step process as it proceeds through a geminal diamine **78** in which both enzyme and substrate amino groups are bonded to C4' (scheme 30). In this structure, the carbon atom has tetrahedral geometry. Both the external and internal aldimines have planar geometry about C4' and their interconversion through the

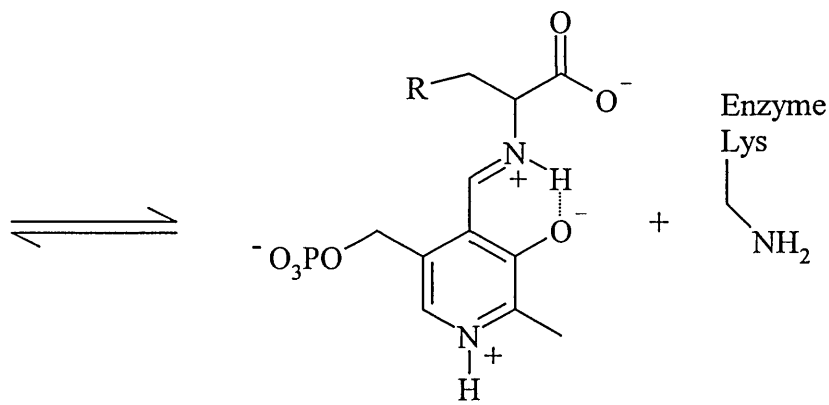
geminal diamine involve changes in geometry as well as proton transfer between the two nitrogens[126].



77 Internal aldimine



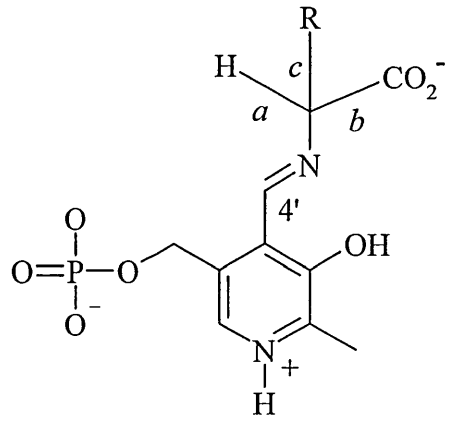
78 Geminal diamine



79 External aldimine

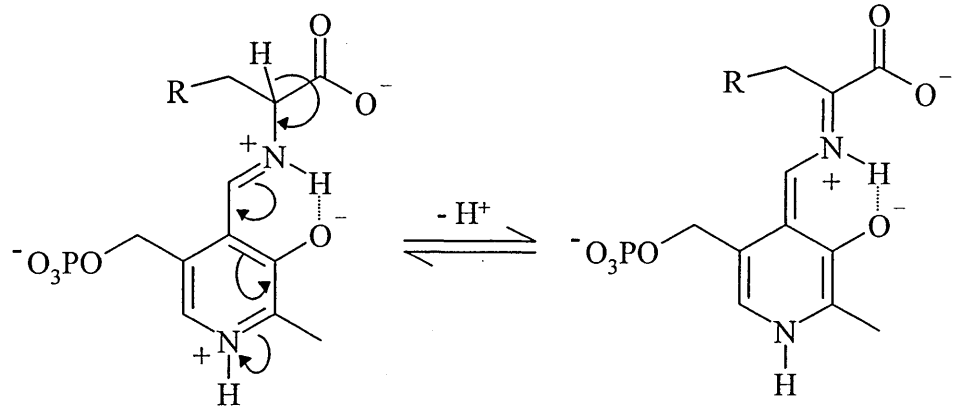
Scheme 30

The next step allows for three possible reactions. The enzymatic reactions catalysed by pyridoxal 5'-phosphate have been shown to be under strict stereochemical control[127]. Depending upon which of the three bonds break in structure **80**, other than the C-N bond to the chiral centre, amino acid can undergo transamination (bond *a* breaks), decarboxylation (bond *b*), or retroaldol reaction (bond *c*).



80

Chemically, an obligatory requirement for electrons to be delocalised between two π-electron systems is that the interacting atomic or molecular orbital components must be coplanar. Pyridoxal 5'-phosphate acts by delocalising electrons through the imine conjugated to the pyridine ring. The pyridine ring defines the appropriate plane, where its π-orbitals can be regarded as composed of overlapping p-orbitals at right angles to the plane of the ring and to become conjugated, the imine p-orbitals must adopt the same orientation[128]. Hence, the electrons that come from the breaking bond *a*, *b*, or *c* must fit into the same plane to be accepted into the π-system of the pyridoxal 5'-phosphate molecule. Loss of any of the three α-substituents results in a resonance stabilised carbanion-quinoniod structure **81,** in scheme 31, which has been shown to be formed in many systems[129].



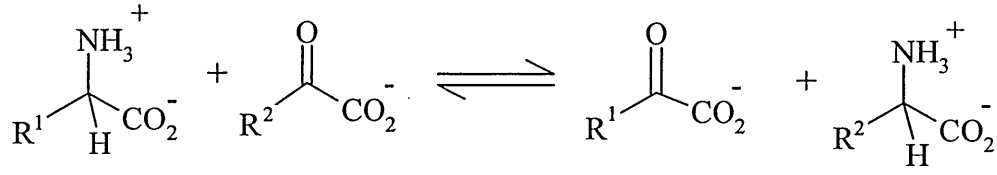
79 External aldimine

81 Quinonoid intermediate

Scheme 31

1.4.2.1. Enzymic transamination and decarboxylation reactions.

Transamination between amino and oxo (keto) acids is catalysed by aminotransferases (scheme 32).



Scheme 32

Aspartate aminotransferase, like all aminotransferases, requires pyridoxal 5'-phosphate as a coenzyme which catalyses the reaction in scheme 33.

PLP-enz + L-Aspartate ⇌ PMP-enz + Oxaloacetate

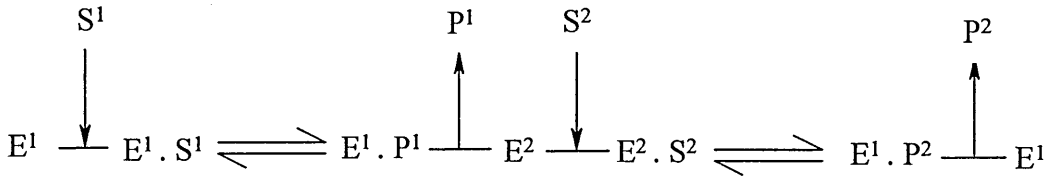
PMP-enz + 2-Oxoglutarate ⇌ PLP-enz + L-Glutamate

Summary : L-Aspartate + 2-Oxoglutarate ⇌ Oxaloacetate + L-Glutamate

Scheme 33

The mechanism of action of this enzyme is essentially similar to the non-enzymic pyridoxal catalysed transaminations. After the initial transaldimination steps common to all pyridoxal 5'-phosphate dependent enzymes, the enzyme abstracts the proton

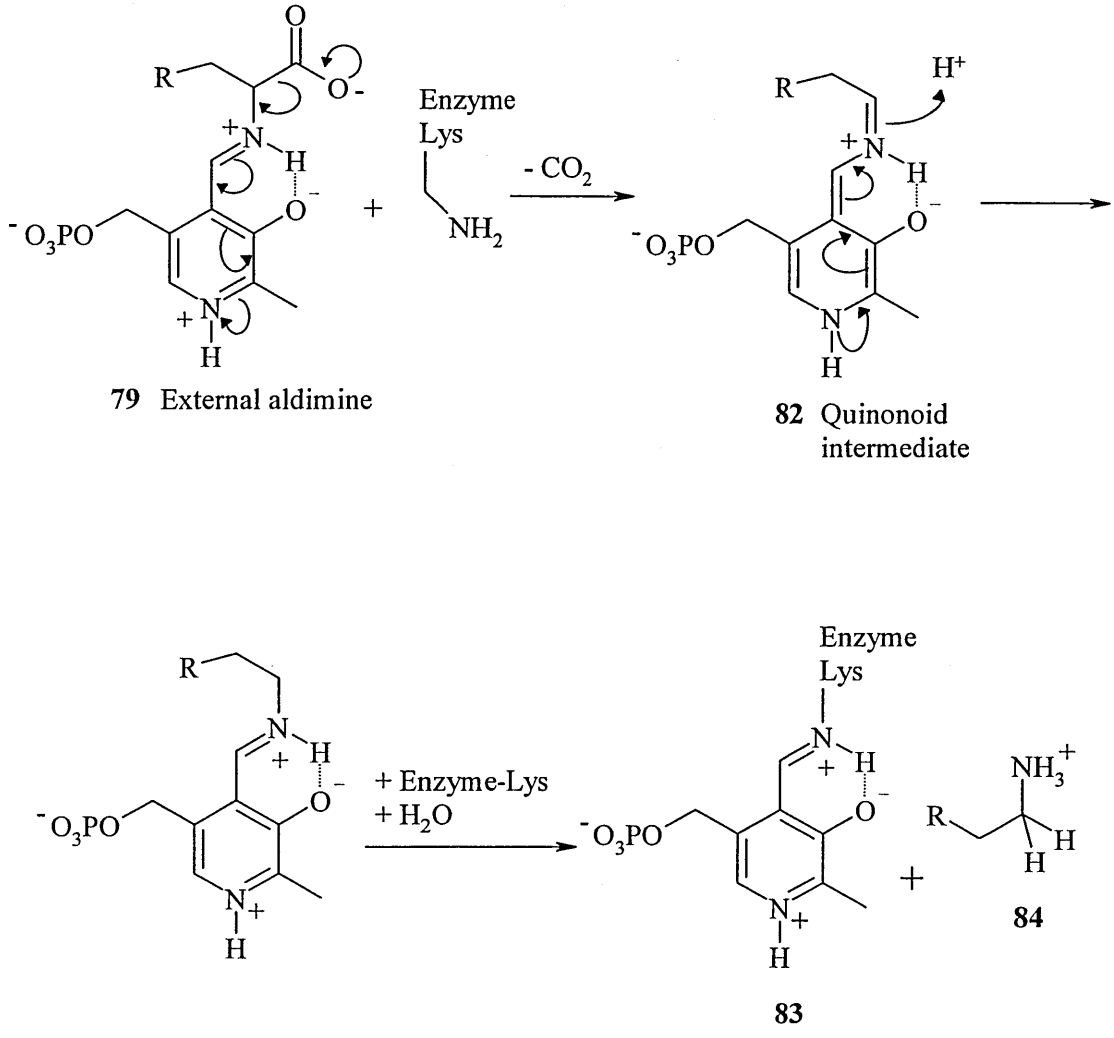
from Cα of the aspartate substrate to form a quinonoid intermediate and subsequent protonation at C4' of the coenzyme. This will liberate the oxaloacetate and the enzyme-bound pyridoxamine remains. The overall reaction is then completed by the occurrence of the reverse process. The enzyme-bound pyridoxamine binds to 2-oxoglutarate which forms the Schiff's base. The Schiff's base is de-protonated at the C4', and then re-protonated at Cα of the amino acid substrate to give L-glutamate. Enzyme-bound pyridoxamines have been isolated as products of the first half reaction of a number of transaminases and the mechanism formulated above corresponds with the 'ping-pong bi-bi' kinetics (scheme 34) which has been observed for many transaminases[130].



E = Enzyme
S = Substrate
P = Product

Scheme 34

In decarboxylation reactions pyridoxal 5'-phosphate is present in enzymes, such as histidine decarboxylase, as an internal aldimine formed with the amino group of a specific residue. This internal aldimine group reacts with the amino acid substrate by transaldimination to form an external aldimine **79**, which through the strongly electrophilic character of its pyridoxylidene moiety weakens the bond to the carboxyl group and results in loss of CO_2. A proton adds to the resulting carbanion **82**, and the product **84** is released (scheme 35). In enzymatic reactions, the incoming proton assumes the stereochemical position vacated by the leaving COOH group. All double bonds of the transition state are thought to lie in a plane that facilitates the indicated electron displacement; for efficient labilization in such a system the bond to the carboxyl group must lie perpendicular to this plane[131].



79 External aldimine

82 Quinonoid intermediate

83

84

Scheme 35

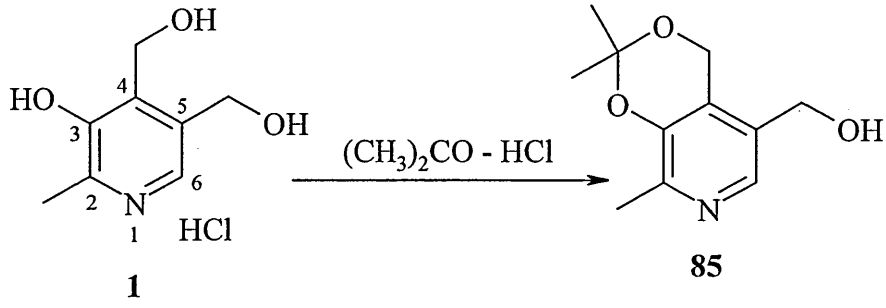
The pyridoxal 5'-phosphate dependent enzymes are an important class of enzymes that catalyse a wide variety of biological transformations involved in the metabolism of amino acids and amines, such as transamination, decarboxylation, racemisation, β- and γ-elimination, and substitution. Although, the catalytical roles of pyridoxal 5'-phosphate are amazingly versatile, its catalytical functions rely primarily on the ability of the coenzyme to act as an electron sink, temporarily storing the electrons that are later used for the cleavage and/or formation of covalent bonds.

1.5. Synthesis of vitamin B_6 analogues.

1.5.1. Manipulation of vitamin B_6 and derivatives.

Manipulation of readily available pyridoxine hydrochloride, through blocking-deblocking procedures, has been utilised for the preparation of vitamin B_6 analogues. This allows one or two functional groups to be blocked using another group such as isopropylidene, benzyl, or acyl, thus permitting the remaining functional groups in the vitamin B_6 molecule to be modified selectively. After suitable modification, the blocking can be removed to give the desired compound. The success of this approach depends on the ease with which the blocking group can be removed and be introduced. Many vitamin B_6 analogues have been prepared by this method as it can conveniently transform selective functional groups of pyridoxine to the desired derivative.

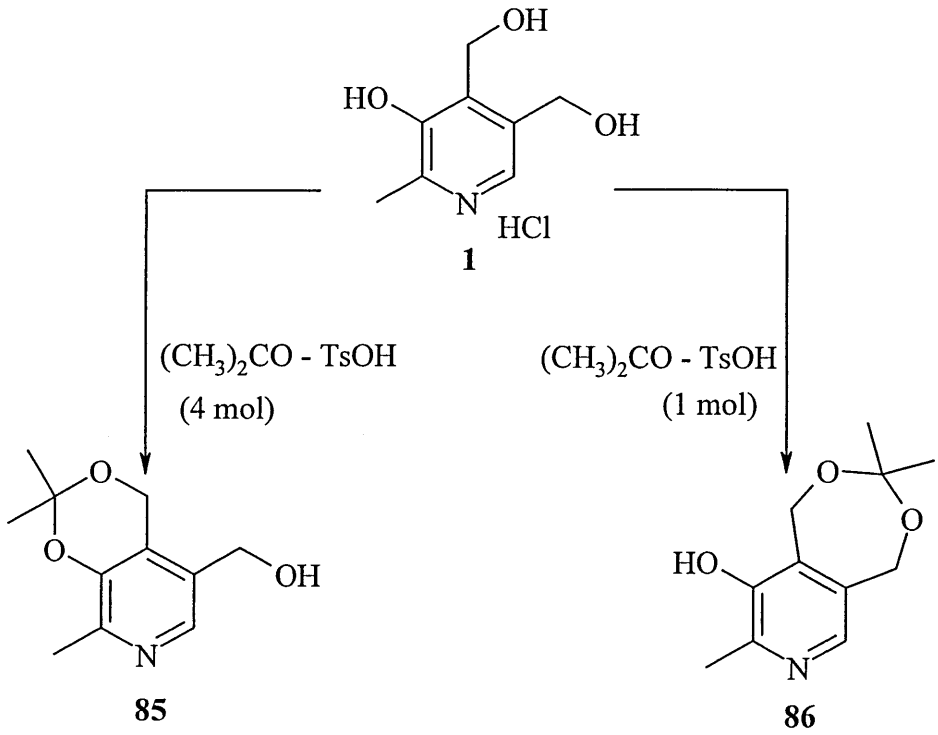
Hence, a typical procedure in pyridoxine manipulation begins with the blocking of C-3, C-4' or C-5' hydroxyl groups. This is done commonly by the addition of an isopropylidene group to C-3 and C-4' or C-4' and C-5' hydroxyl groups of pyridoxine. The treatment of pyridoxine **1** in acetone saturated with hydrochloride gas[132] is generally used to achieve the 3,4'-*O*-isopropylidenepyridoxine **85** (scheme 36).



1 **85**

Scheme 36

On the other hand, treatment of pyridoxine with equimolar amount of *p*-toluenesulphonic acid in dimethoxypropane and acetone produces 4',5'-*O*-isopropylidenepyridoxine **86** exclusively. However, if the *p*-toluenesulphonic acid is in a 4:1 ratio to pyridoxine then 3,4'-*O*-isopropylidenepyridoxine **85** was the only product[132], as shown in scheme 37.

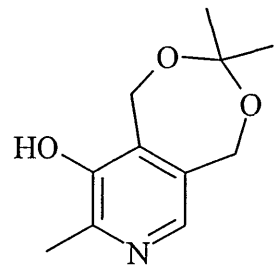


Scheme 37

The 4',5'-*O*-isopropylidenepyridoxine **86** was considered as the initial product even when a large proportion of the catalyst was used and that the formation of **86** is followed by isomerisation to 3,4'-*O*-isopropylidenepyridoxine **85**. Apparent isomerisation of **86** to **85** has been reported to occur in acetone with HCl[133]. Isomerisation was examined in an experiment using labelled material 4',5'-*O*-isopropylidene-d_6-pyridoxine[132], prepared by reacting pyridoxine **1** with acetone-d_6. The labelled 4',5'-*O*-isopropylidene-d_6-pyridoxine was subjected to 'isomerisation procedure' using *p*-toluenesulphonic acid in acetone and revealed that isolated 3,4'-*O*-isopropylidenepyridoxine **85** was unlabelled. The compound **85** was unlabelled suggest that the 'isomerisation procedure' first causes the isopropylidene group in **86** to be completely hydrolysed off, and then the cyclic ketal **85**, with a six-membered ring, is formed directly. This is probably a case of kinetic versus thermodynamic control, with compound **86** as a kinetic product and **85** as a thermodynamic product. However, increase in acidity favours the formation of ketals involving the phenolic OH group than to those involving only the alcoholic OH groups.

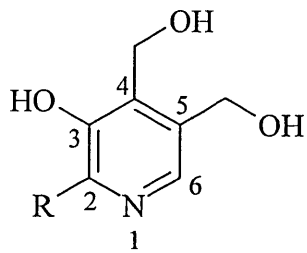
1.5.1.1. Reaction at the 2-position.

The 4',5'-*O*-isopropylidenepyridoxine **86** is particular useful in selective modification of pyridoxine at the 2-position.



86

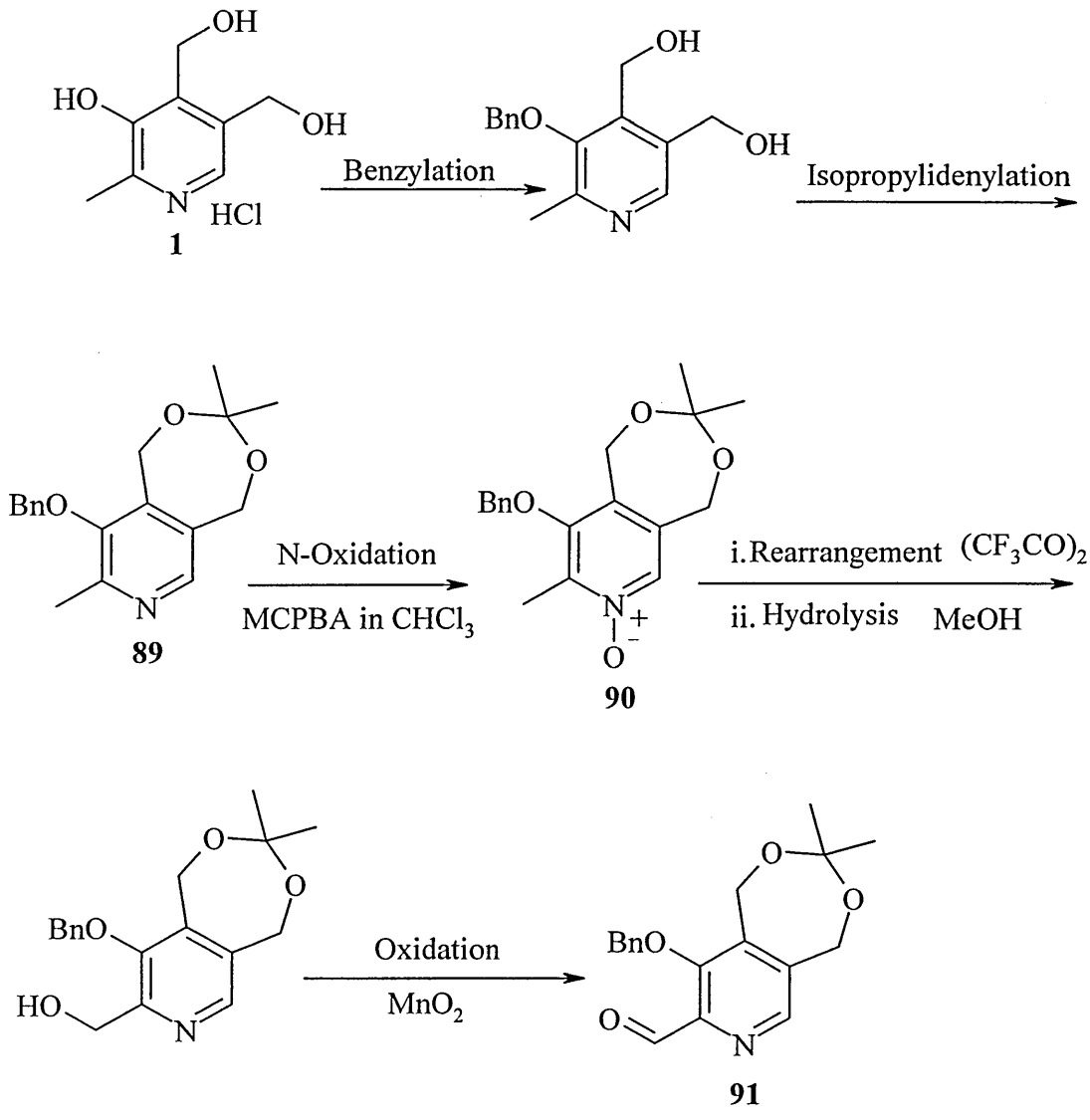
Analogues of vitamin B_6 modified in the 2-position, such as 2'-methylpyridoxine (**87**, R = C_2H_5), have been of considerable biochemical interest regarding pyridoxal catalysis[17], the mode of binding of the coenzyme analogue[134], and in studies of the active sites of enzymes metabolising vitamin B_6[135]. Studies with analogues that had the 2-methyl group replaced with 2-CH_2OH (**88**, R = CH_2OH) or with various other alkyl groups indicated that there is a certain bulk tolerance in the 2-position with respect to several enzymes requiring pyridoxal 5'-phosphate and in metabolic interconversions of different forms of vitamin B_6[1,136].



87 R = C_2H_5

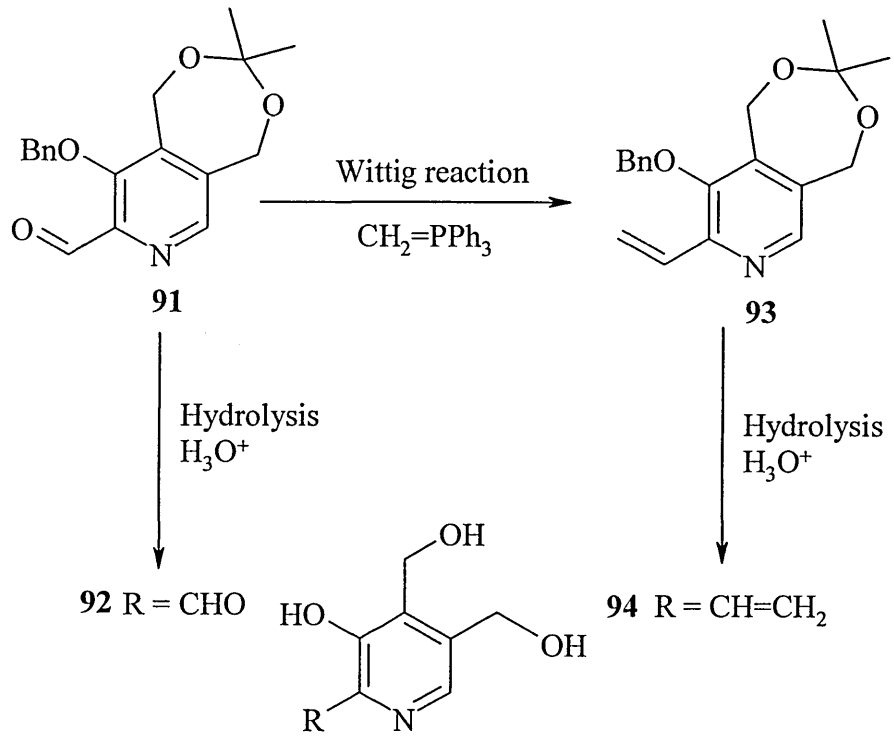
88 R = CH_2OH

A general example of modifying the 2-methyl group of pyridoxine **1** proceeds by protecting the C-3 phenolic group by benzylation followed by isopropylidenylation of the C-4' and C-5' hydroxyl groups to give the derivative **89**. Protection of the derivative's nitrogen by N-oxidation allows modification of the 2-position without interference. The compound **90**, treated with trifluoroacetic anhydride and subsequent hydrolysis of the 2'-*O*-trifluoroacetyl group followed by oxidation with MnO_2 gives the 2-formyl group **91**, as shown in scheme 38.



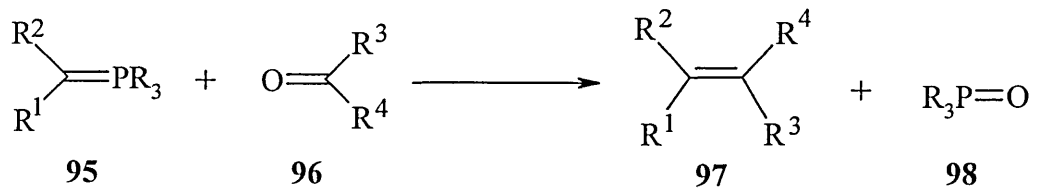
Scheme 38

The blocking groups of compound **91** removed by mild hydrolysis, heating with 1 *N* HCl on a steam bath, will afford 2-formyl-2-norpyridoxine (**92**, R = CHO). The 2-formyl group in **91** can be converted into vinyl derivative **93** by means of a Wittig reaction in which the required ylide can be generated from triphenylphosphonium bromide, using either *n*-butyllithium or potassium *tert*-butoxide as the base, and subsequent mild acid hydrolysis gives 2-vinyl-2-norpyridoxine **94** (scheme 39).



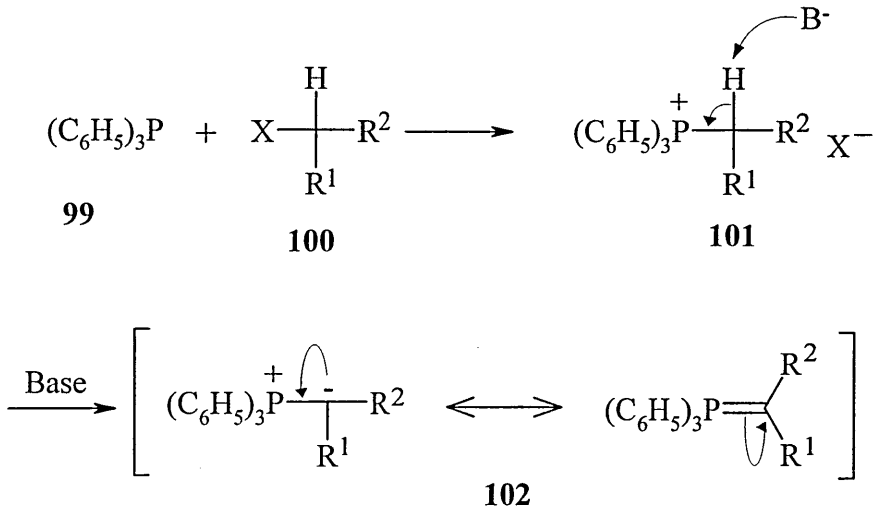
Scheme 39

The Wittig reaction or Wittig olefination[137,138] reaction is the interaction of an alkylidene phosphorane **95** (i.e. a phosphorus ylide) with an aldehyde or ketone **96** to yield an alkene **97** (i.e. an olefin) and a phosphorus oxide **98** (scheme 40).



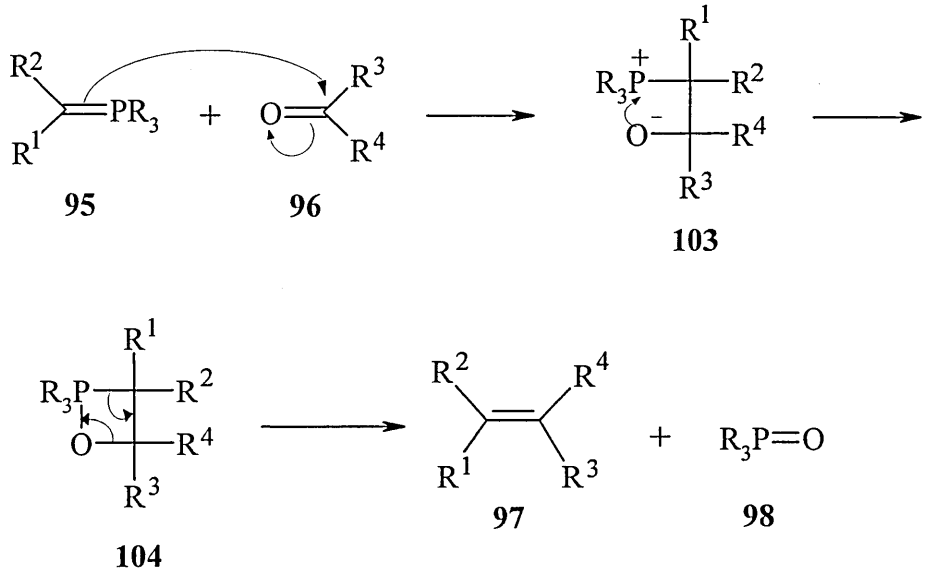
Scheme 40

The most common route in preparing phosphorus ylides, like the triphenylphosphonium bromide used with carbonyl **91**, is the reaction of triphenylphosphine **99** with an alkyl halide **100** to give a triphenylphosphonium salt **101**. The triphenylphosphonium salt **101** treated with a base give the corresponding ylide **102** (scheme 41). The triphenylphosphonium salt **101** is usually isolated, and in most cases is crystalline, while the ylide **102** is usually prepared in solution and used directly for reaction with the carbonyl substrate.



Scheme 41

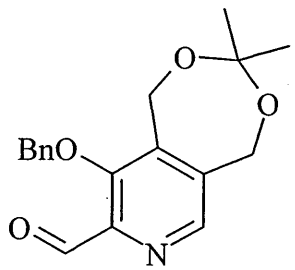
Nucleophilic addition of the negatively polarised ylide carbon centre **95** (due to its resonance structure as in **102**) to the carbonyl carbon centre of an aldehyde or ketone **96** is the initial step of olefin formation. A betaine **103** is thus formed, which can cyclise to give the oxaphosphetane **104** as an intermediate. The latter decomposes to yield a trisubstituted phosphine oxide **98** and the alkene **97** (as shown in scheme 42). The driving force for the reaction is the formation of the strong double bond between phosphorus and oxygen.



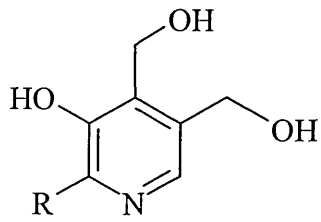
Scheme 42

The reactivity of the phosphorus ylide **95** strongly depends on its substituents (R^1 and R^2). When R^1 or R^2 is an electron-withdrawing group, the negative charge can be delocalised over several centres, and the reactivity at the ylide is reduced. The

reactivity of the carbonyl compound towards addition of the ylide increases with the electrophilic character of the C=O group. R^1 and R^2 are often both alkyl, or alkyl and aryl. The Wittig reaction is one of the most important reactions in organic synthesis. The synthetic importance of the Wittig reaction and its variants and related reactions is based on the fact that the new carbon-carbon double bond in the product molecule is generated at a fixed position.



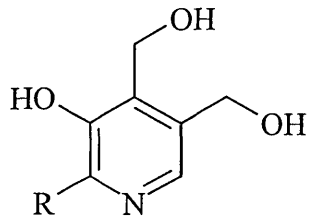
91



92 R = CHO

94 R = CH=CH_2

Both 2-formyl-2-norpyridoxine **92** and 2-vinyl-2-norpyridoxine **94** have shown to be inhibitors of mouse mammary adenocarcinoma cells in culture and the inhibition was not reversed by pyridoxine[132]. The availability of useful intermediates, such as the 2-formyl derivative **91**, for modification of the 2-position made it possible to introduce other substituents as shown in table 2.



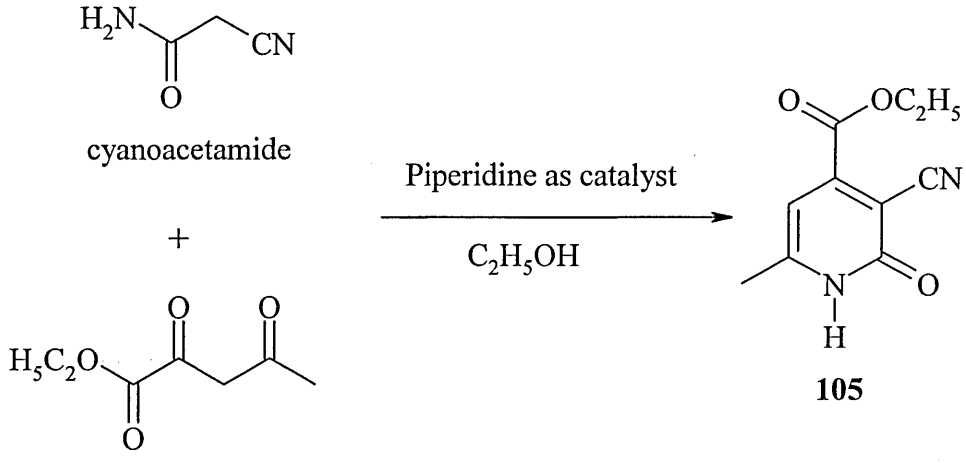
Compound	Biological Activity
2-Ethynyl-2-norpyridoxine[132]; R = C≡ CH α^2-Aminopyridoxine[139]; R = CH_2NH_2 2-Amino-2-norpyridoxine[139]; R = NH_2 2-Norpyridoxine-2-carboxamide[139]; R = $CONH_2$ 2-Chloro-2-norpyridoxine[139]; R = Cl	Inhibits mouse mammary adenocarcinoma cells in culture.
2-Norpyridoxine-2-carboxylic acid[139]; R = CO_2H	None

Table 2

1.5.1.2. Reaction at the 3-position.

Modification of the 3-position has been of much less interest, as the 3-hydroxyl group of the coenzyme of vitamin B_6 appears to be insignificant for binding to apoenzyme since 3-*O*-methylpyridoxal 5'-phosphate binds efficiently to apoaspartate transaminase[112]. Established methods in modifying the 3-position of vitamin B_6 used acyclic precursors as the starting materials[140,141,142]. Since these methods were concerned with modifying the 3-position to a halogen or thiol group, pyridoxine were inappropriate as a starting material due to complication in substituting the hydroxyl group at the 3-position.

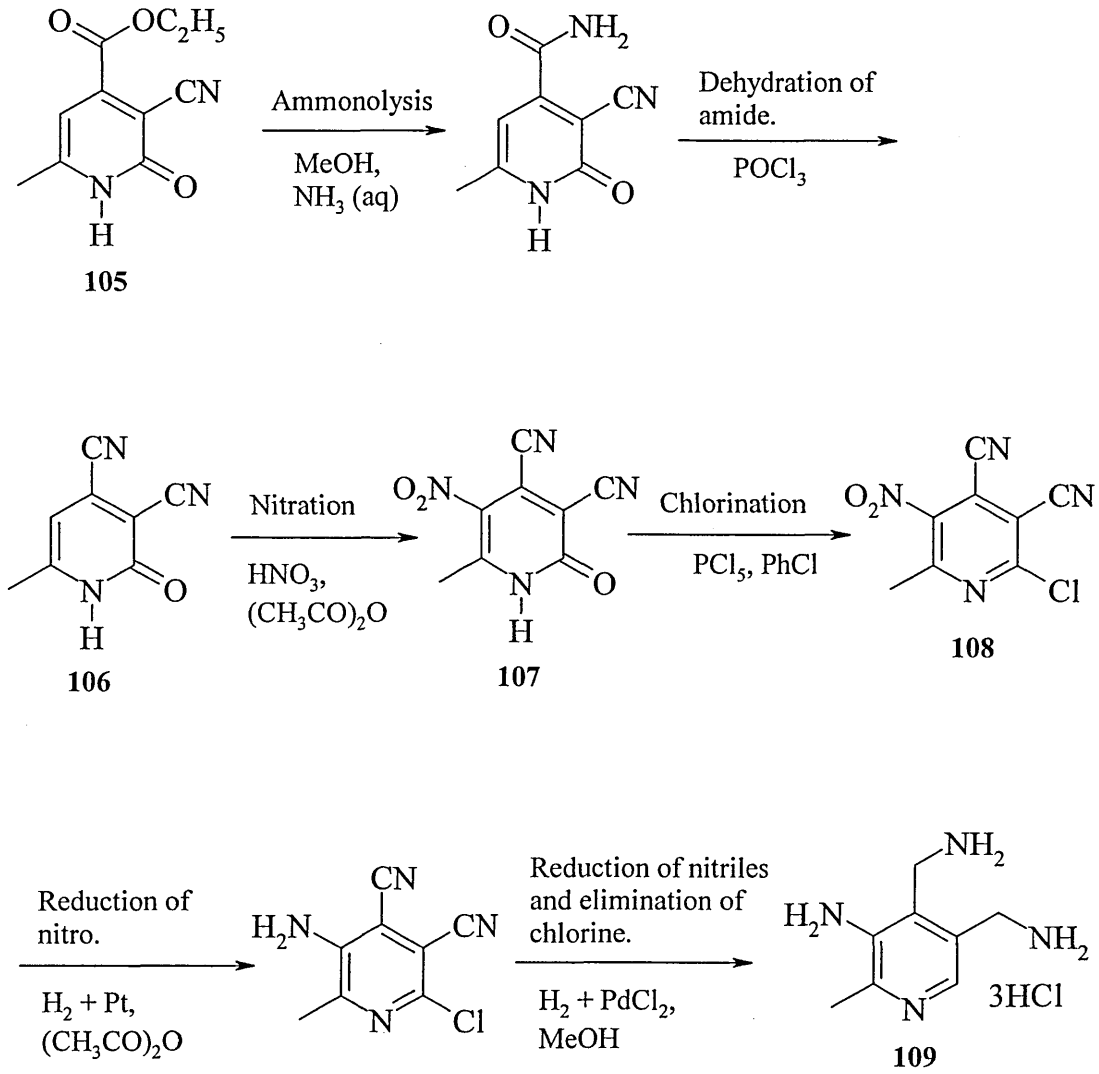
Method for achieving modification at the 3-position are based on the established strategy of total synthesis using acyclic precursors to form the pyridine derivative for subsequent substitution reactions. Hence, condensation of cyanoacetamide and 2,4-dioxopentanoic acid ethyl ester[141, 143] forms 3-cyano-4-carboethoxy-6-methyl-2-pyridone **105** (scheme 43).



Scheme 43

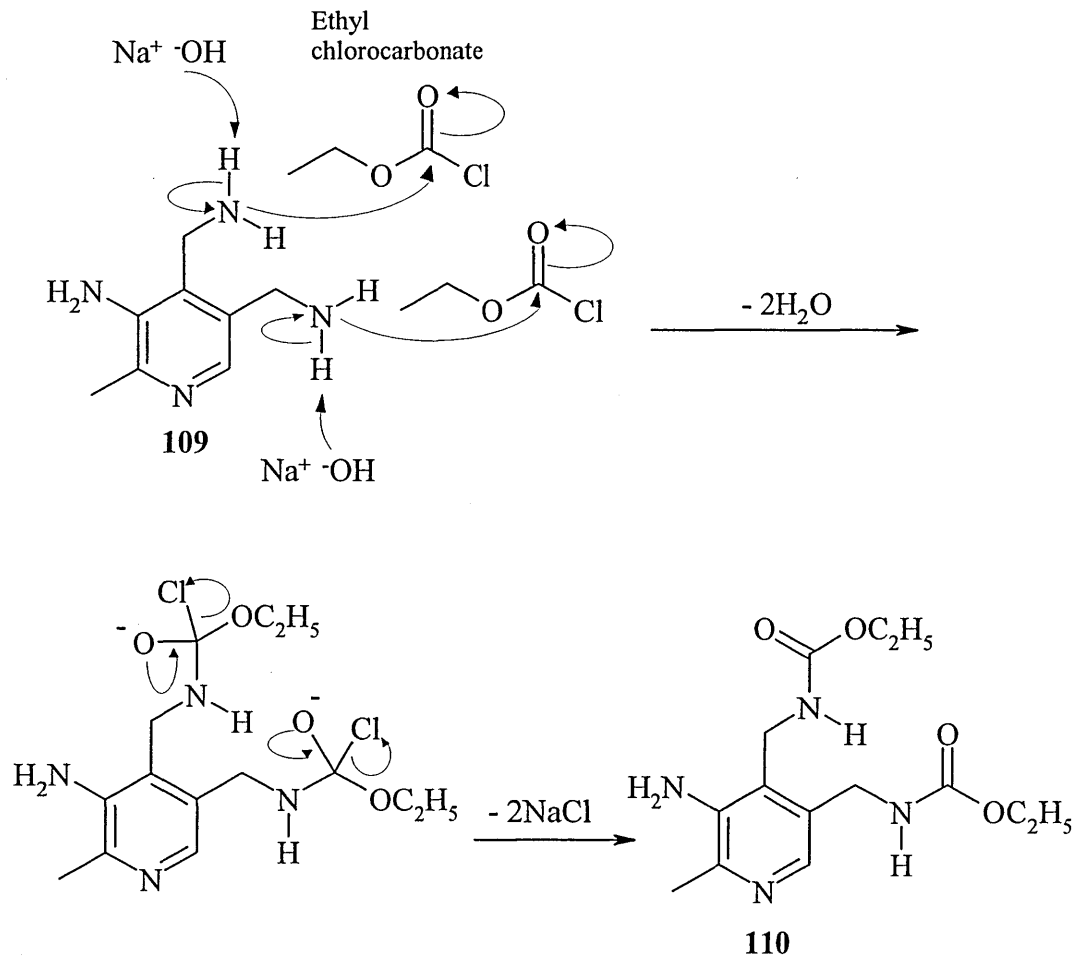
Subsequent ammonolysis of pyridone **105** and dehydration of the obtained amide using phosphorous oxychloride afforded dinitrile **106**. Nitration of the dinitrile **106** using fuming nitric acid in acetic anhydride forms nitropyridone **107**. The nitropyridone **107** was subjected to phosphorus pentachloride in chlorobenzene to yield the chlorinated pyridine **108**. Reduction of the nitro group by catalytic hydrogenation using platinum oxide as catalyst and subsequent conversion of the

resulted nitriles to amines by catalytic hydrogenation using palladium catalyst afforded triamine **109**, as shown in scheme 44.



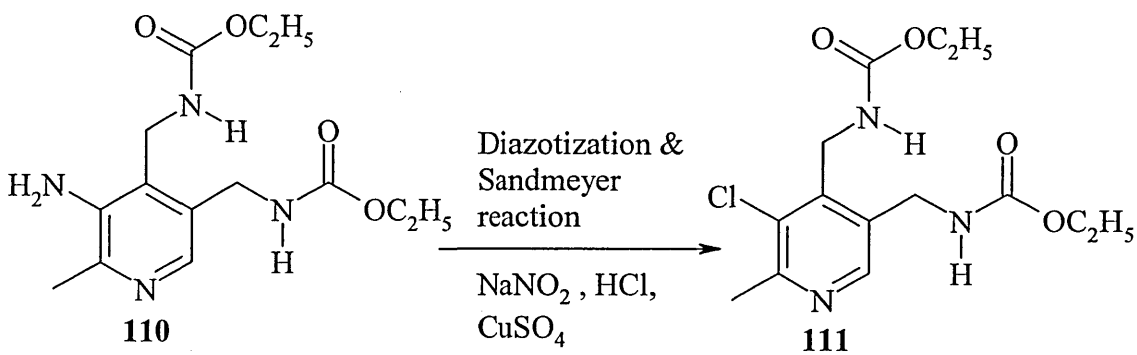
Scheme 44

Subsequent blocking of the two aliphatic amino groups at the 4- and 5-position in compound **109** allows selective substitution of the 3-amino group to a chloro substituent. The two aliphatic amino groups at the 4- and 5-position undergoes carbethoxylation[144], where the nucleophilic amines attacks the carbonyl carbon atom of ethyl chlorocarbonates and the chlorines dissociate to form the carbamate **110**, as shown in scheme 45.



Scheme 45

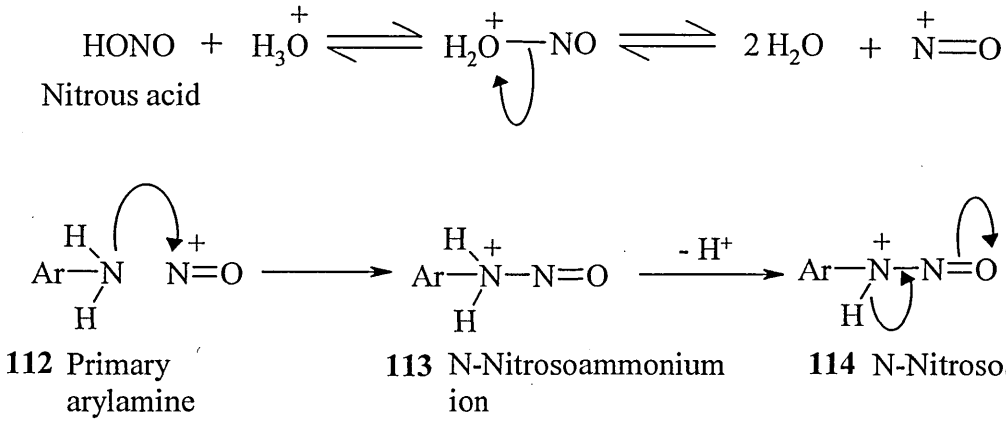
The compound **110** undergoes diazotization which results in the replacement of the 3-position diazonium group with a chloro substituent, to form derivative **111** (scheme 46).



Scheme 46

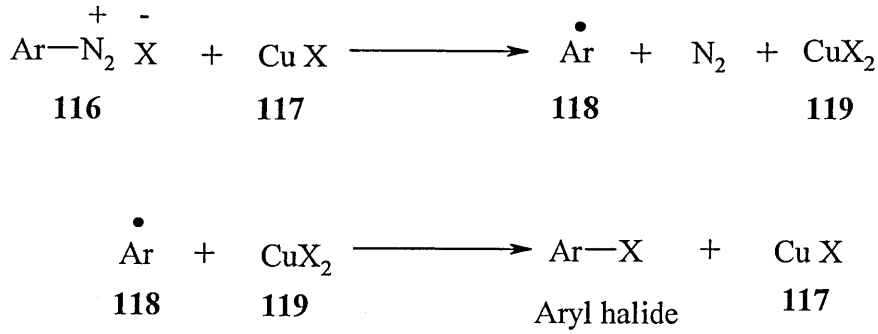
Diazotization[145] of compound **110** illustrates the most important reaction of amines with nitrous acid. Compound **110**, like primary arylamines, reacts with nitrous acid to give arenediazonium salts. The general mechanism proceeds in the presence of strong

acid, when nitrous acid dissociates to produce ^{+}NO ions which react with the nitrogen of the amine **112** to form an unstable *N*-nitrosoammonium ion **113**. The *N*-nitrosoammonium ion loses a proton to give an *N*-nitrosoamine **114**, tautomerises to a diazohydroxide **115** and subsequently loses water to form the diazonium ion **116**, as shown in scheme 47.



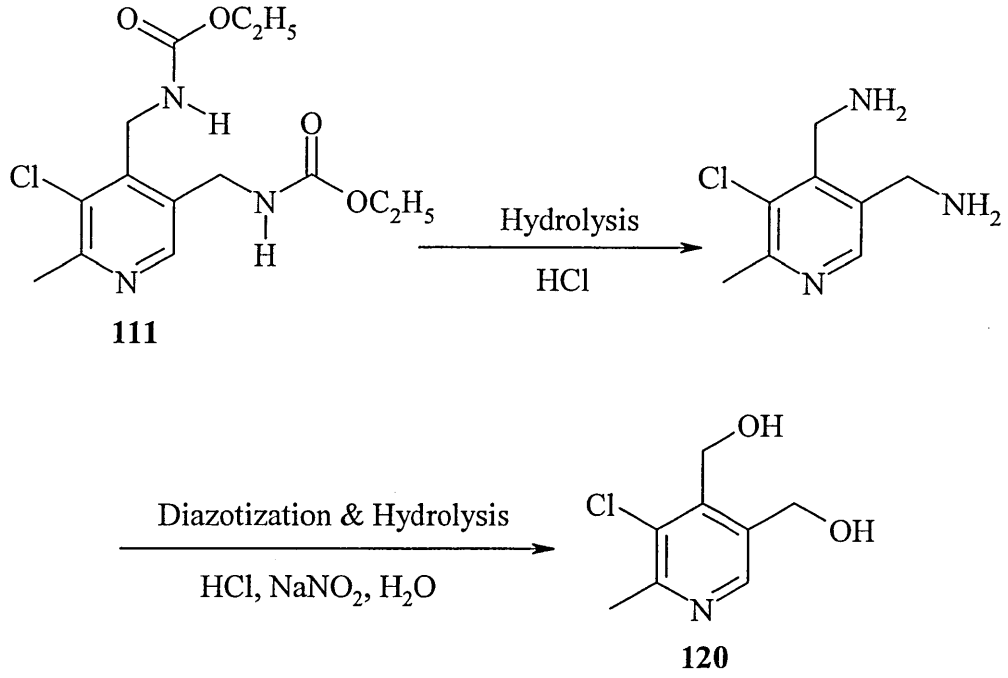
Scheme 47

Subsequent replacement of the diazonium group in e.g. **116** with a chloro substituent is known as the Sandmeyer reaction[146]. The Sandmeyer reaction is used for the replacement of the diazonium group in an arenediazonium compound by halide or pseudohalide, taking place in the presence of a metal salt (with the exception of iodide which is possible without a metal catalyst). The general reaction mechanism involves the diazonium ion **116** being reduced by the reaction with copper-(I) salt **117** to give an aryl radical **118**. This radical then abstracts a halogen atom from the CuX_2 **119**, which is thus reduced to the copper-(I) salt **117**. Since the copper-(I) species is regenerated, it serves as a catalyst in the overall reaction (scheme 48).



Scheme 48

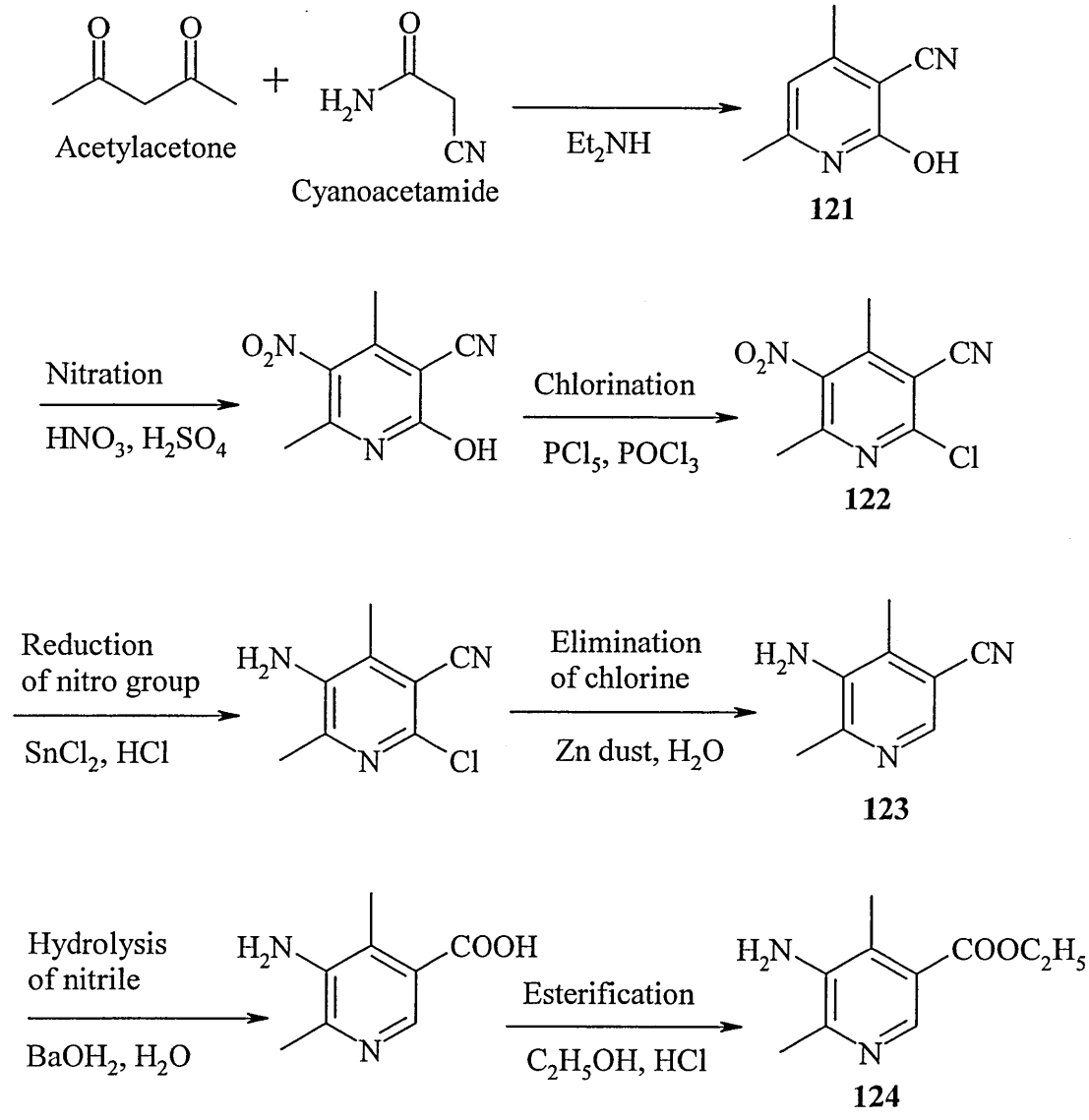
The obtained chloro compound **111** can be hydrolysed to remove the amino blocking groups at the 4- and 5-position to provide pyridoxamine derivative. Further diazotization and hydrolysis of the pyridoxamine derivative will give the pyridoxine analogue **120** with a chloro group at the 3-position as shown in scheme 49.



Scheme 49

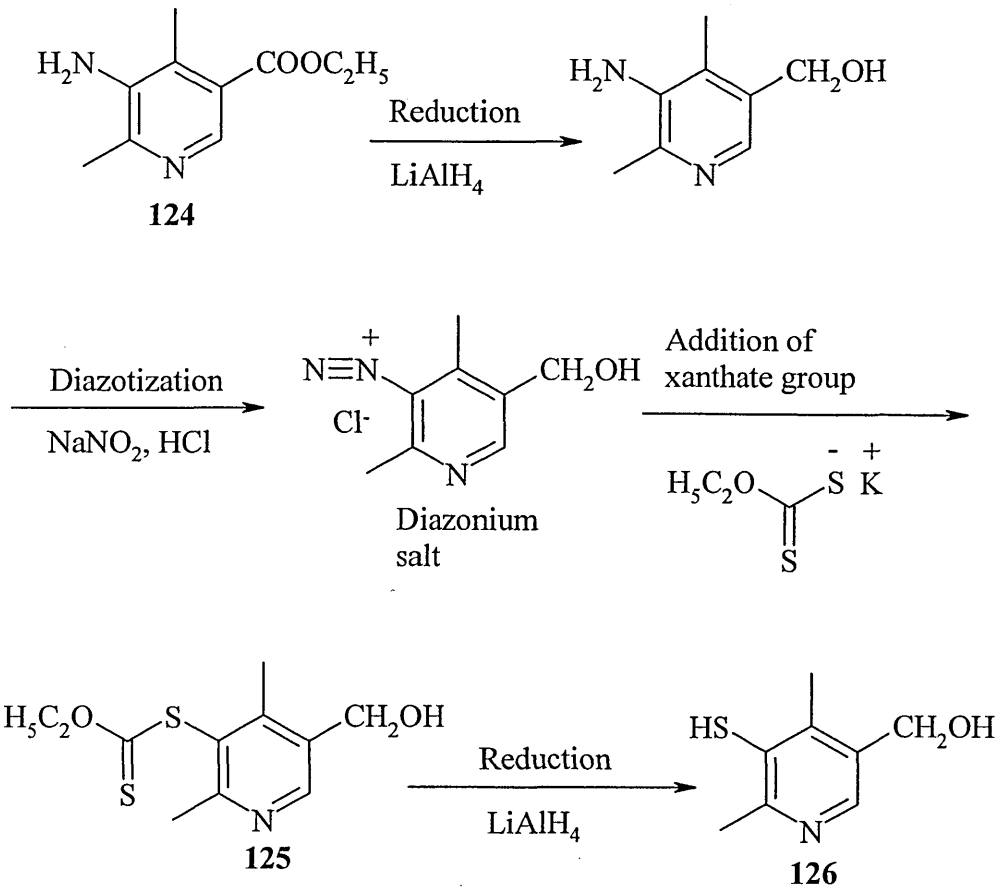
Another example involves the condensation of cyanoacetamide and acetylacetone[142] to form 2,4-dimethyl-5-cyano-6-hydroxypyridine **121**. Nitration of pyridine **121** and subsequent conversion of the 6-position hydroxyl group to a chloro substituent yields compound **122**. Reduction using tin(II) chloride in hydrochloric acid converted the nitro in compound **122** to an amino group, and subsequent treatment with zinc dust removed the chloro substituent to give aminonitrile compound **123**. The compound **123** treated with barium hydroxide hydrolysed the

nitrile group to the corresponding carboxyl group and esterification using ethanolic hydrogen chloride produced the ester compound **124**, as shown in scheme 50.



Scheme 50

Reduction of the ester in compound **124** using lithium aluminium hydride and subsequent diazotization gives the corresponding diazonium intermediate. The diazonium intermediate was treated accordingly with potassium ethyl xanthate to form the xanthate **125**. Subsequently, the xanthate ester was reduced with lithium aluminium hydride to produce the thiol compound **126**, as shown in scheme 51.

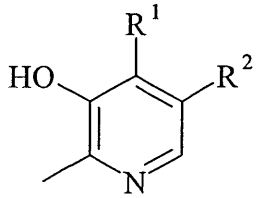


Scheme 51

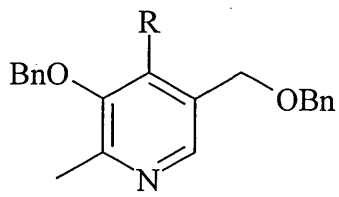
Compounds **120** and **126** with the 3-position hydroxyl group replaced with chloro and thiol substitutents, respectively, does not have significant effect on the implicated biological systems[140,142].

1.5.1.3. Reaction at the 4-position.

A majority of vitamin B_6 analogue syntheses have focused on manipulation of the 4-position, since the most important part of the active pyridoxal 5'-phosphate is the 4-position aldehyde group, where its main function is to bind to an amine substrate and form a Schiff's base which initiates subsequent catalytic reaction in biological systems. Thus, most vitamin B_6 analogues were prepared in order to mimic or antagonize the catalytic ability of the biologically active form of vitamin B_6[1]. The replacement of the 4-position aldehyde group with an inert methyl group, the 4-deoxypyridoxine (7, $R^1 = CH_3$; $R^2 = CH_2OH$), has resulted in the classical and most widely studied antagonist of vitamin B_6[6].



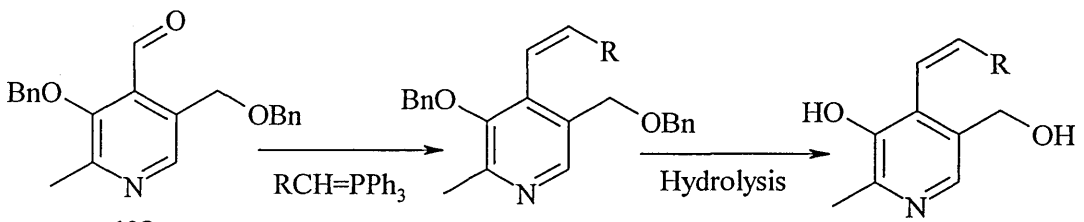
7



127 R = CH_2OH

128 R = CHO

The most general and convenient method for the modification of the 4-position begins with the compound 3,5'-*O*-dibenzylpyridoxine **127** or 3,5'-*O*-dibenzylpyridoxal **128**; the synthesis of these will be detailed in later discussion. The 3,5'-*O*-dibenzylpyridoxal **128** undergoes a variety of substitution reactions to modify the 4-position without affecting neighbouring functional groups. For instance, the application of the Wittig reaction to derivative **128** affords compound **129**. The subsequent removal of the benzyl groups either by heating with dilute hydrochloric acid or, in the case of sensitive groups, with neat trifluoroacetic acid, produces the 4-vinyl-4-deformylpyridoxal (**132**, R = H) [147] and several of its α,β-unsaturated derivatives[4,148] as shown in scheme 52. These substituted vinyl analogues were found to be active as inhibitors of mouse mammary adenocarcinoma cells in culture[148].



128

$RCH{=}PPh_3$

129 R = H
130 R = CH_2CH_3
131 R = CH = CH_2

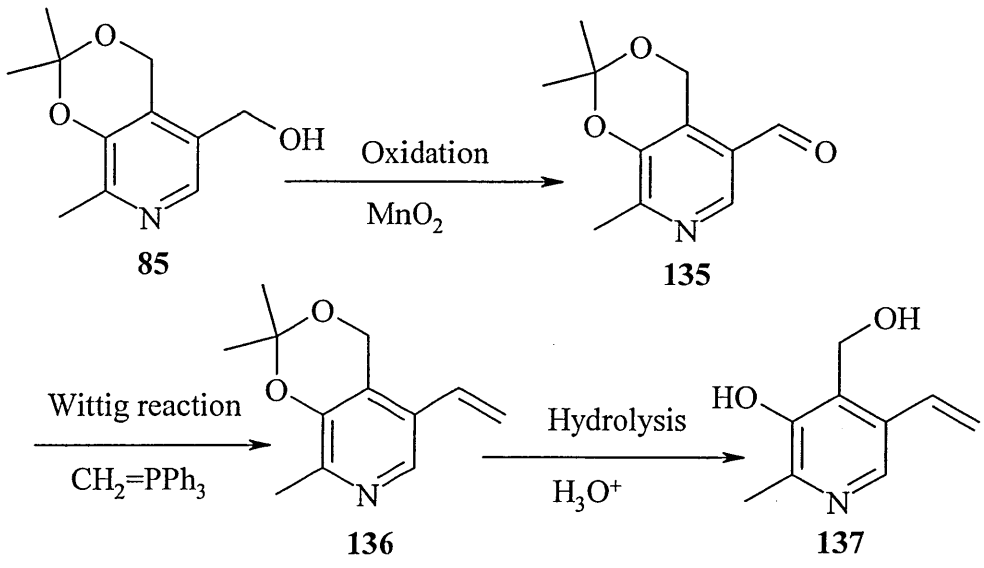
Hydrolysis

132 R = H
133 R = CH_2CH_3
134 R = CH = CH_2

Scheme 52

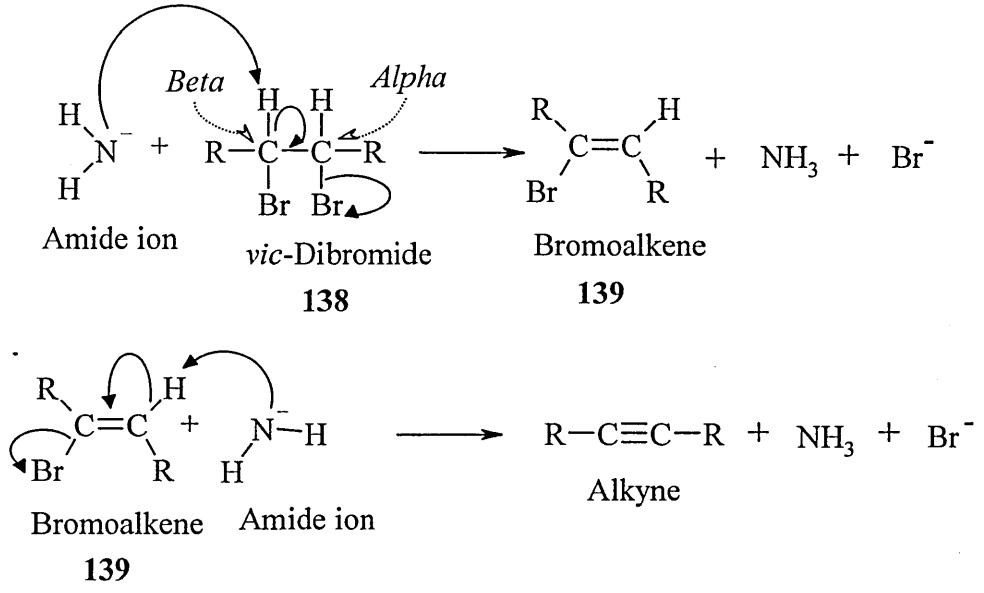
1.5.1.4. Reaction at the 5-position.

Modifications at the 5-position are more accessible, primarily because of the ease of preparation of the key compound, 3,4'-isopropylidenepyridoxine **85**. It is possible that the phosphate group has a direct function in enzymic catalysis or that it plays an essential structural role in enzymes, thus various analogues with modified side chains in the 5-position were synthesised[149,150]. For example[151], oxidation of the 3,4'-isopropylidenepyridoxine **85** gives the 5-aldehyde compound **135** which undergoes a Wittig reaction to achieve the 5-vinyl compound, followed by mild acid hydrolysis to produce the 5-vinyl pyridoxine derivative **137** (scheme 53).



Scheme 53

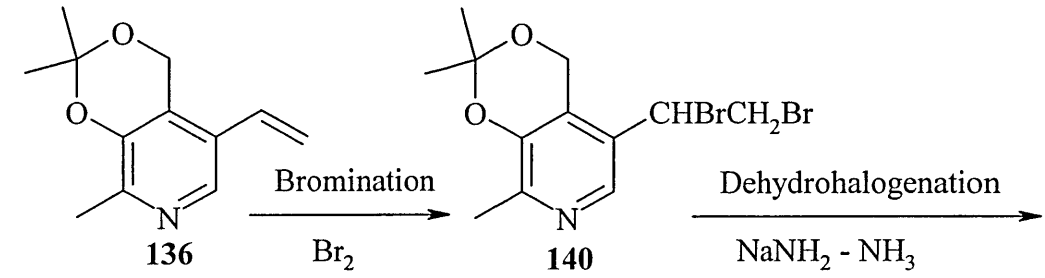
Alternatively, alkynes can be synthesised from the alkene by bromination to form a *vic*-dibromo compound **138**. The *vic*-dibromo compound is dehydrohalogenated through its reaction with a strong base. The dehydrohalogenation occurs in two steps, and the first step yields a bromoalkene **139** as shown in scheme 54. The basic ion brings about an E2 reaction. The E2 reaction proceeds by the removal of a proton from the 'β-carbon' whilst at the same time the electron pair of the β C-H bond begins to move in to become the π-bond of a double bond. Subsequently the bromine begins to depart with the electrons that bonded it to the 'α-carbon'. A second E2 reaction produces the alkyne.



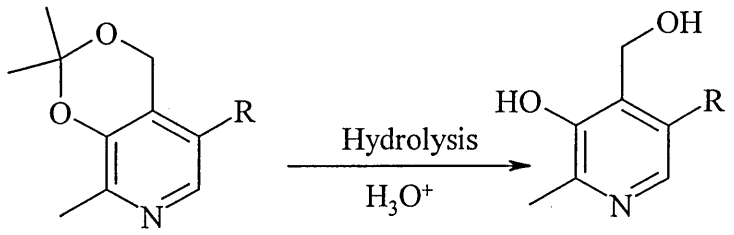
Scheme 54

Depending on the conditions, these two dehydrohalogenations may be carried out as separate reactions, or they may be carried out consecutively in a single reaction. The strong base, sodium amide, is capable of producing both dehydrohalogenations in a single reaction.

Thus, bromination of the 5-vinyl compound **136** forms the 5-(dibromoethyl) derivative **140**, which eliminates the bromide ions through dehydrohalogenation to give bromoalkene **141** and alkyne **142**. Hydrolysis of the isopropylidene groups on compounds **141** and **142** gives the pyridoxine analogues **143** and **144**, respectively (scheme 55).



136 **140**



141 R = CH=CHBr

142 R = C≡CH

143 R = CH=CHBr

144 R = C≡CH

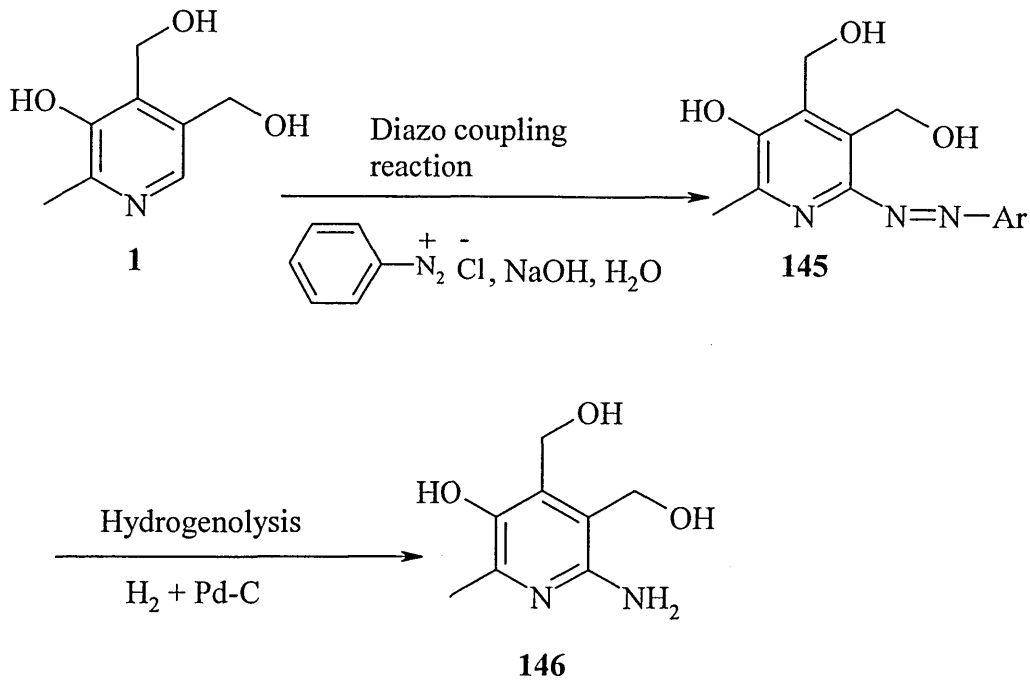
Scheme 55

The 5-vinyl **137**, 5-bromovinyl **143**, and 5-ethynyl analogues **144** have all exhibited the ability to act as substrates of pyridoxine dehydrogenase[151].

1.5.1.5. Reaction at the 6-position.

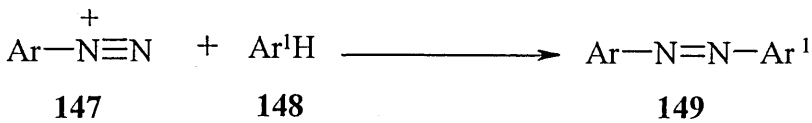
Halogenation at the 6-position is one of the established routes in synthesising vitamin B_6 analogues. In early studies[152], the greater electron-withdrawing character of the 6-halogen as compared with hydrogen offered an opportunity to correlate these factors with their effects on enzymes, cells, and tissues dependent on vitamin B_6 for their functions. The general method starts with 6-aminopyridoxine **146** which was obtained by hydrogenolysis of 6-phenylazopyridoxine **145**. The 6-

phenylazopyridoxine[153] **145** can be prepared by the coupling reaction of pyridoxine **1** with benzenediazonium chloride (scheme 56).



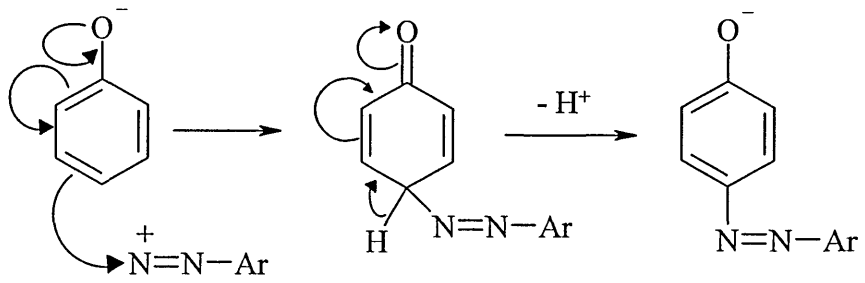
Scheme 56

This electrophilic aromatic substitiution is often called a diazo coupling reaction[154]. The arenediazonium ions **147** undergo a coupling reaction with electron-rich aromatic compounds **148** like aryl amines and phenols to yield azo compounds **149** (scheme 57).



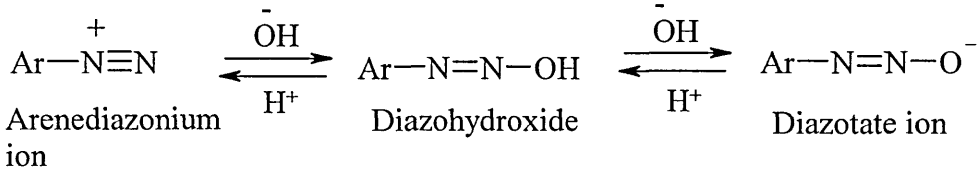
Scheme 57

The substitution reaction at the aromatic system **148** usually takes place *para* to the activating group. If the *para* position is already occupied by a substituent, the new substitution takes place *ortho* to the activating group. Therefore, phenols (like pyridoxine **1**) are predominantly coupled in slightly alkaline solution, in order to first convert an otherwise unreactive phenol into the reactive phenoxide anion which initiates the coupling reaction, as shown in scheme 58.



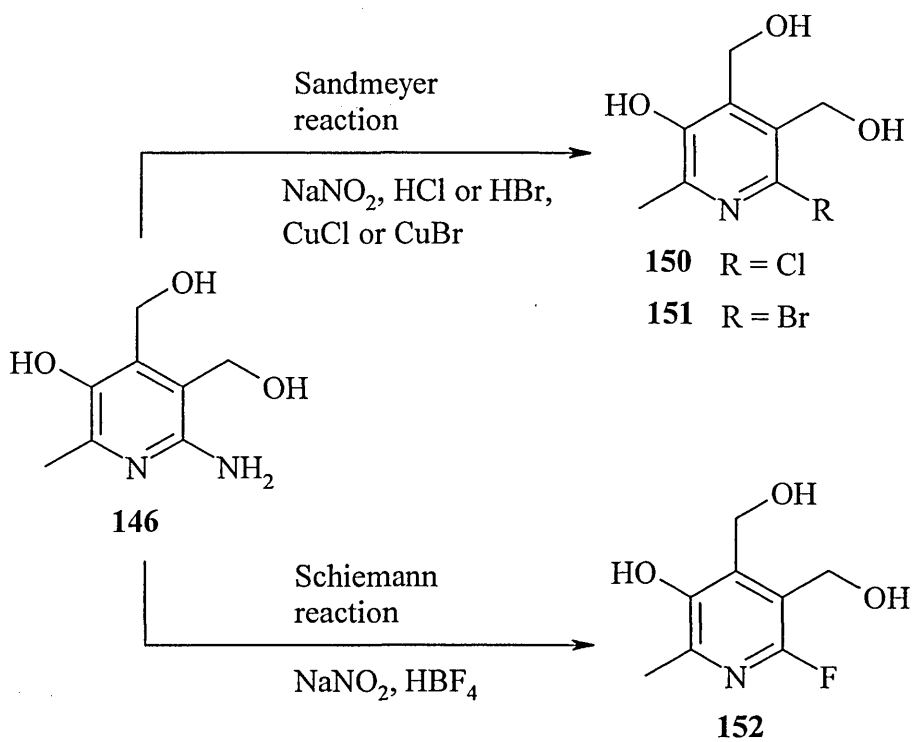
Scheme 58

If the solution is too alkaline (pH > 10) the arenediazonium ion itself reacts with hydroxide ion to form a relatively unreactive diazohydroxide or diazotate ion, shown in scheme 59.



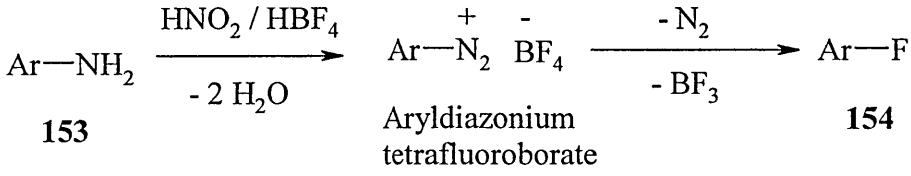
Scheme 59

The 6-aminopyridoxine **146** can undergo the Sandmeyer reaction to yield 6-chloro **150** or 6-bromopyridoxine **151**. Alternatively, the 6-amino compound **146** undergoes the Schiemann reaction to afford the 6-fluoro **152** (scheme 60).



Scheme 60

The Schiemann reaction [155] is very similar to the Sandmeyer reaction. The diazotization of aryl amine **153** in the presence of tetrafluoroborate leads to formation of an aryldiazonium tetrafluoroborate that can be converted into aryl fluoride **154** by thermolysis (scheme 61).



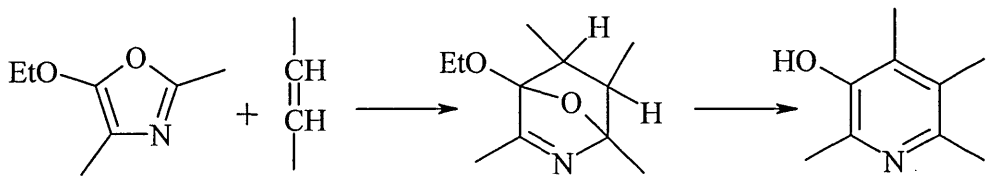
Scheme 61

Only the 6-amino **146** and the 6-fluoro **152** analogues were found to be active as inhibitors of mouse mammary adenocarcinoma cells in culture. Also, the 6-chloro compound **150** showed that with the bulky substituent it precludes the compound from being phosphorylated by pyridoxal kinase[152].

Since the discovery that vitamin B_6 exists in multiple forms, and that pyridoxal 5'-phosphate has coenzyme functions in various enzyme systems, various compounds related to vitamin B_6 have also been synthesised. The modification strategies mentioned are just some of the possibilities in achieving vitamin B_6 analogues. Alterations have been made at every position of the pyridine ring and the effects of these alterations on diverse biological and biochemical systems have been extensively studied[1,149].

1.5.2. Synthesis of vitamin B_6 analogues by the condensation of oxazoles with dienophiles.

In the late 1950's, it was discovered[156] that the azadiene system of oxazoles readily reacts with an activated double bond by the Diels-Alder mechanism. Since then, the Diels-Alder reaction of oxazoles **155** with dienophiles **156** has been applied extensively to the synthesis of substituted pyridine bases [157] **157** (scheme 62), including biologically important compounds such as vitamin B_6 and its analogues.

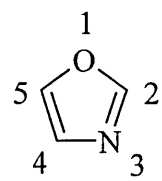


155 **156** **157**

Scheme 62

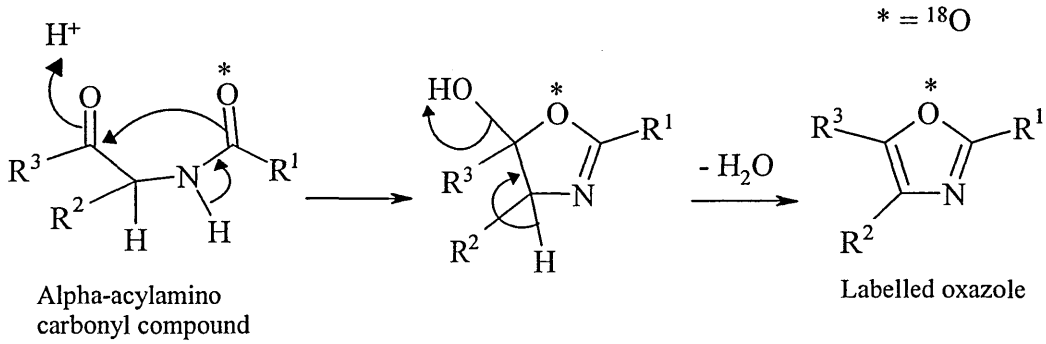
1.5.2.1. Oxazoles: synthesis.

Oxazole **158** has an oxygen atom and a pyridine-type nitrogen atom at the 1- and 3-position of the ring. The oxazoles are planar molecules with conjugated π-electron sextets in the cyclic system. Their chemical properties are aromatic in character. The lone-pair of electrons on nitrogen, which is coplanar with the heterocyclic ring and therefore not involved in delocalisation, present weakly basic properties as in pyridine.



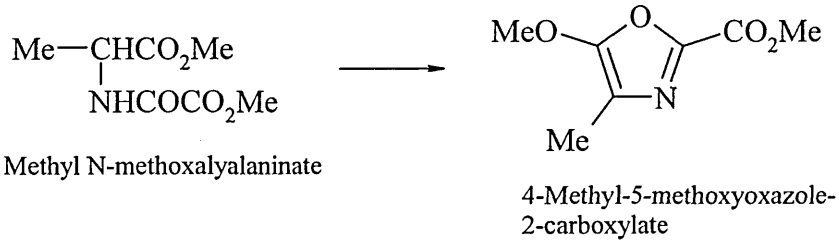
158

One of the oldest and most useful syntheses of oxazoles is the reaction of α-acylamino carbonyl compounds with a cyclodehydrating agent, such as PCl_5, H_2SO_4, $SOCl_2$, or $POCl_3$. The cyclodehydration reaction is known as the Robinson-Gabriel synthesis[158,159]. The mechanism of the cyclodehydration was uncovered when it was found that labelling the α-acylamino ketones with ^{18}O in the keto carbonyl group did not produce ^{18}O-labelled oxazoles. Conversely, when the amide carbonyl oxygen is labelled with the ^{18}O, the label is found in the oxazole (scheme 63)[160].



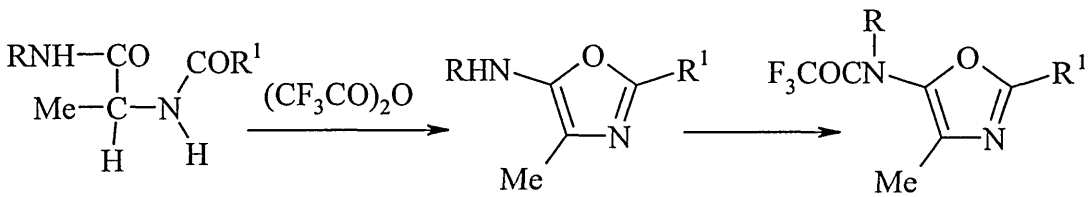
Scheme 63

In many cases when the cyclodehydrating agents are PCl_5, H_2SO_4, $SOCl_2$, or $POCl_3$, the Robinson-Gabriel synthesis affords low yields of oxazoles. However, when polyphosphoric acid is used, the yields of oxazoles can be increased to 50 – 60 %[161]. Anhydrous HF was also shown to efficiently cyclise *N*-aryl-α-amido ketones to oxazoles in yields up to 96 %[162]. Also, methyl *N*-methoxalyalaninate treated with phosgene in triethylamine at room temperature for 10 min produced 4-methyl-5-methoxyoxazole-2-carboxylate in 80 % yield[163] (scheme 64).



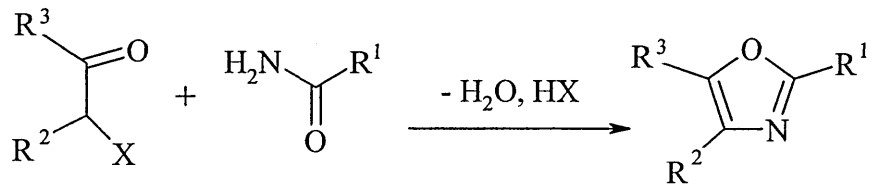
Scheme 64

Also, acetic and trifluoroacetic anhydride have been utilised as cyclodehydrating agents for the conversion of secondary amides to oxazoles[164] (scheme 65).



Scheme 65

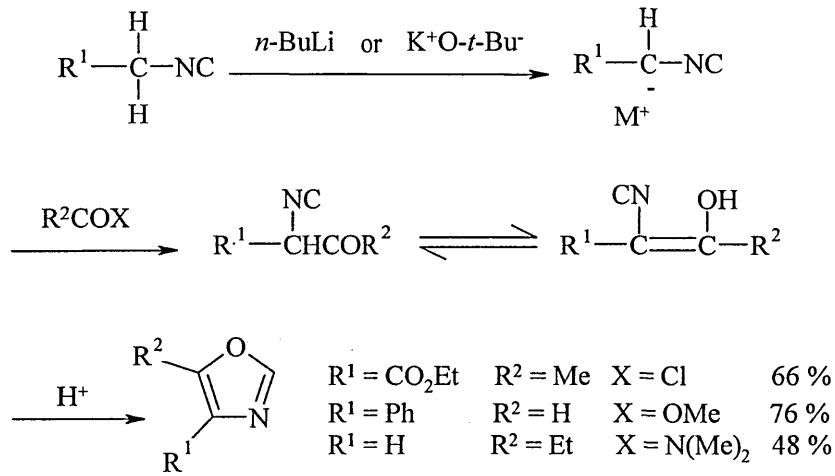
Including the Robinson-Gabriel synthesis, there are various methods for oxazole synthesis. α-Haloketones and α-hydroxyketones condense with acid amides via an *O*-alkylation, to give 4- and 5-substitued oxazoles, but often in low yields (scheme 66)[165].



Scheme 66

The synthesis also suffers from a lack of generality. Whereas 4- and 5-alkyl- or aryloxazoles can be prepared using this reaction, oxazoles bearing other functional groups at these positions generally cannot be synthesised. Often the relative inaccessibility of the starting α-functionalised ketone may be a major downside to the use of this method.

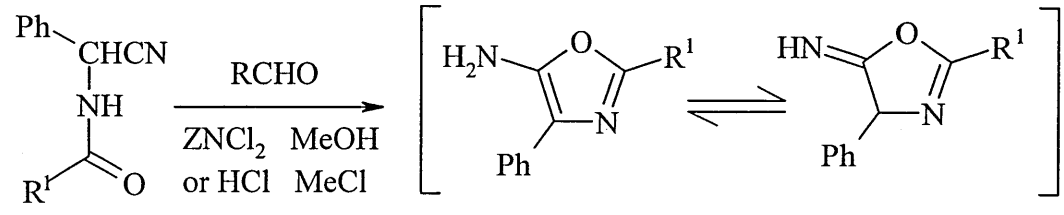
The use of isocyanides overcomes disadvantages associated with obtaining appropriate α-substituted ketones. The reaction of α-metalated isocyanides with carboxylic acid derivatives, via *C*-acylation and electrophilic C-O bond formation, produced 2-unsubstituted oxazoles in moderate to high yield[166,167](scheme 67).



Scheme 67

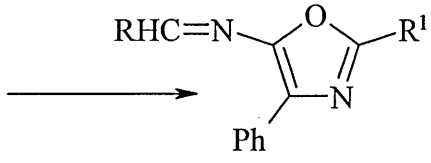
A number of variations of this method have been developed. The commercially available tosylmethyl isocyanide (TOSMIC) and derived reagents add the CH-N=CH fragment to unsaturated substrates (e.g. aldehydes) to give oxazoles in good yield[168].

Synthesis which utilise amino-, imino-, and amido-nitriles produce a wide range of 5-substituted oxazoles from reactions with aldehydes[169]. The acid-induced cyclisation of α-amidonitriles yields the 5-aminooxazoles and subsequent reaction with aromatic aldehydes forms the 5-arylideneaminooxazoles (scheme 68)[170].



Alpha-amidonitrile compound

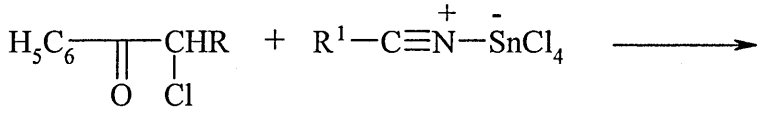
5-Aminooxazole derivative



5-Arylideneaminooxazole

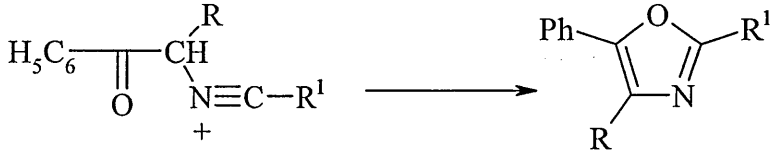
Scheme 68

Nitrilium salts, generated by the reaction of the nitrile-$SnCl_4$ complex with α-haloketone, cyclise to produce 2,4-disubsituted-5-phenyloxazoles (scheme 69)[169]. The reaction is general for the R = Ph, R^1 = alkyl, alkoxyalkyl, benzyl, or aryl and give yields of 52 – 92 %.



Alpha-haloketone

Nitrile-$SnCl_4$ complex

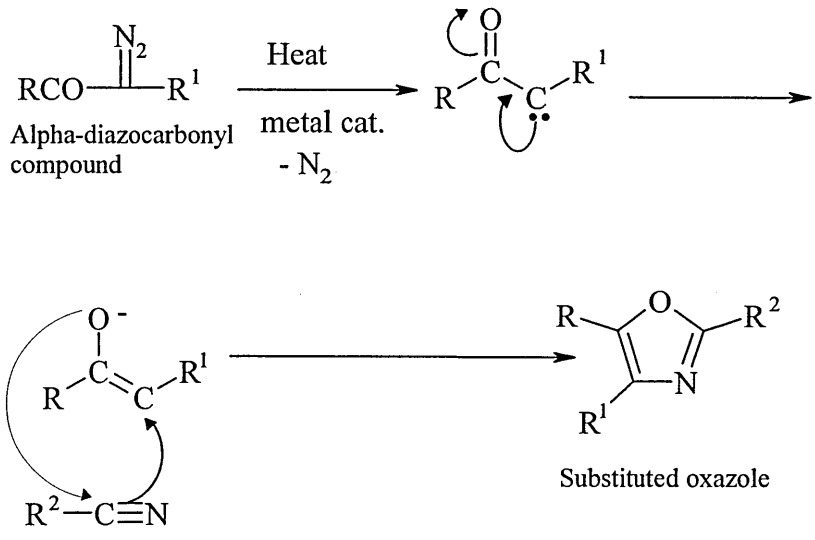


Nitrilium salt

2,4-Disubstituted-5-phenyloxazole

Scheme 69

Low yields of oxazoles can be obtained from carbonylcarbenes as shown in scheme 70[169]. Upon heating, the diazo compound releases N_2 to give the ionic intermediate, which reacts with the nitrile to form the desired product.

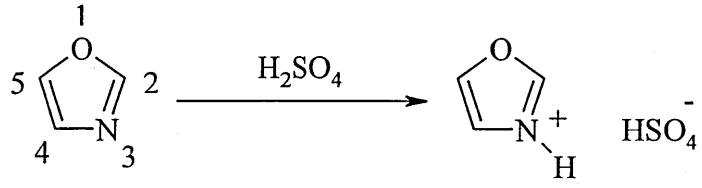


Scheme 70

Although there are a variety of methods to choose from for oxazole synthesis, the Robinson-Gabriel synthesis is the most well known and convenient to use.

1.5.2.2. Oxazoles: reactions.

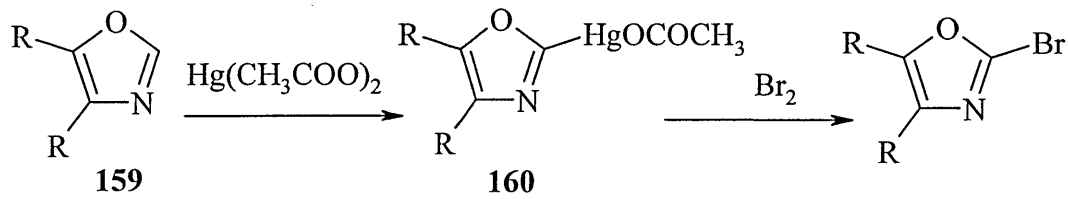
The electronegativity of the pyridine-like nitrogen atom causes the π-electron density to be low, especially on the C-2 atom[169]. As oxazoles **158** are weak bases, strong acids (e. g. H_2SO_4) tend to protonate the N-atom resulting in oxazolium salts, which react faster with nucleophiles than oxazoles (scheme 71).



Scheme 71

Electrophiles attack oxazoles preferentially at the 5-position, then the 4-position. The oxazole ring system does not undergo electrophilic attack readily unless electron-releasing substituents are present. Although electrophilic substitution reactions are possible with oxazoles, they are frequently accompanied by the addition reactions, as in furan. Bromination and mercuration are the most general electrophilic substitution reactions of oxazoles. Mercury (II) acetate in acetic acid acetoxymercurates 4-substituted oxazoles in the 5-position, 5-substituted oxazoles in

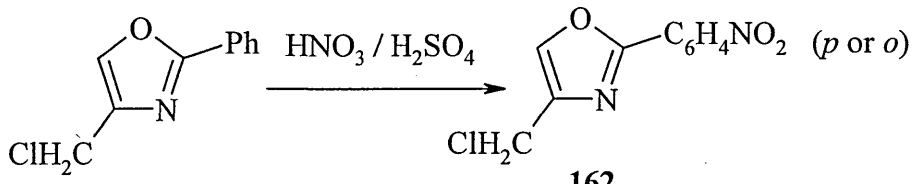
the 4-position, and 4,5-disubstituted oxazoles **159** in the 2-position. The acetoxymercury group **160** can be substituted by electrophiles such as bromine, as shown in scheme 72.



159 **160**

Scheme 72

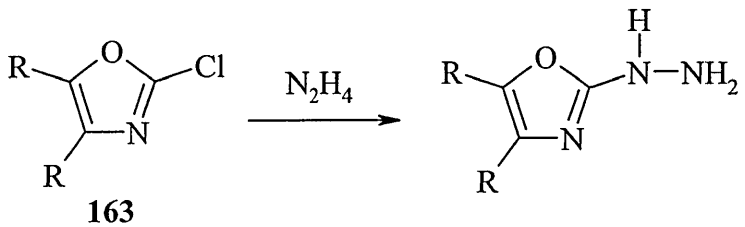
In general, the pyridine-like N-atom in the oxazole impedes electrophilic substitution reactions. This is evident when phenyloxazoles **161** are nitrated in the benzene ring rather than in the heterocycle giving oxazole **162** (scheme 73).



161 **162**

Scheme 73

Nucleophilic substitution reactions on the oxazole ring are relatively uncommon; it will only occur if certain functional groups are present. Because of the low π-electron density on the C-2 atom, nucleophilic substitution reactions of 2-halo-oxazoles proceed rapidly. Thus, treatment of 2-chlorooxazole **163** with hydrazine readily affords the substituted hydrazine (scheme 74). The ease of nucleophilic displacement of halogens is hal-2 > hal-4 > hal-5.

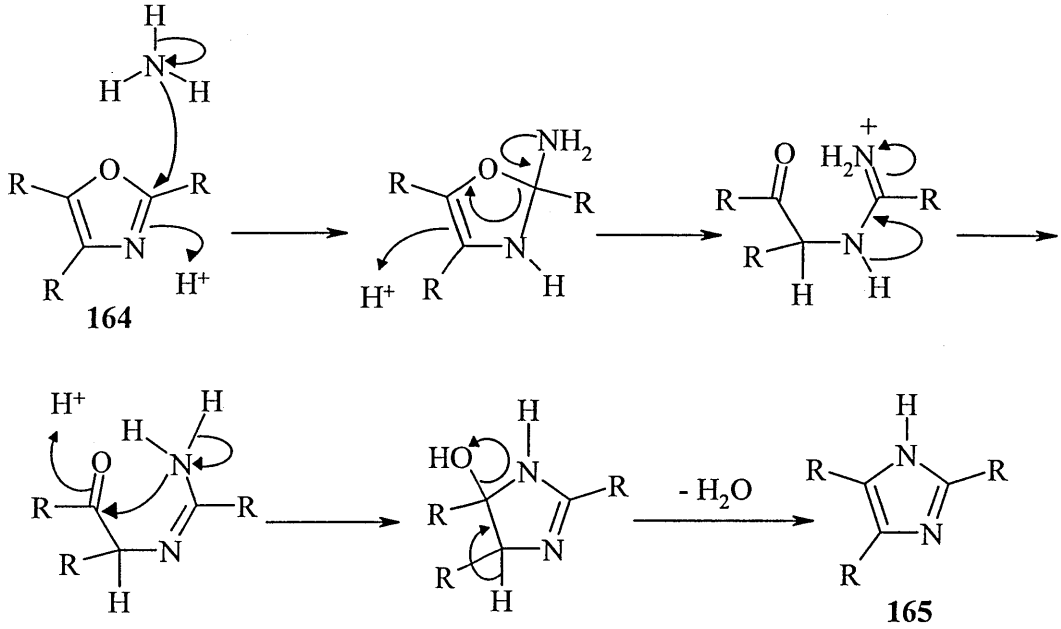


163

Scheme 74

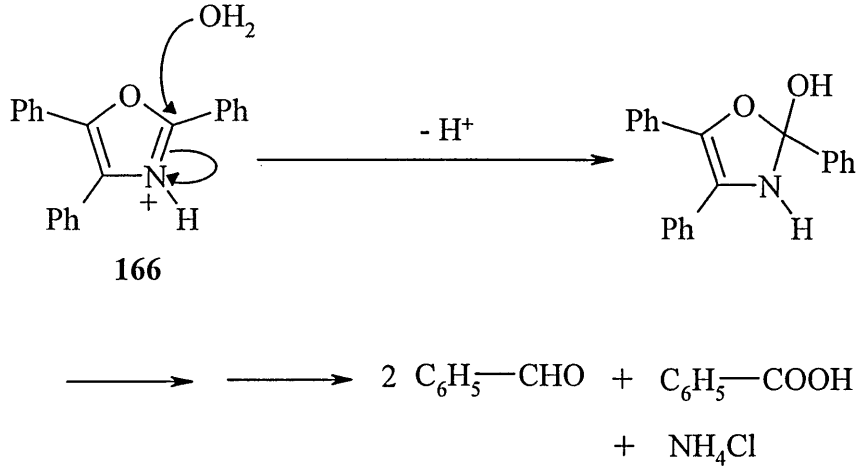
Cleavage of the oxazole ring by nucleophilic attack is a much more common reaction than nucleophilic substitution reactions. The initially formed acyclic intermediates derived from this type of ring cleavage may be stable toward further reactions, and in some instances, the acyclic product undergoes a subsequent cyclisation reaction

whereby a new ring is formed[165]. The structure of this new system is largely dependent upon the structure of the attacking nucleophile and on the functional groups on the oxazole. For example, on heating with ammonia, formamide or primary amines, oxazoles **164** undergo a ring transformation into imidazoles **165** (scheme 75).



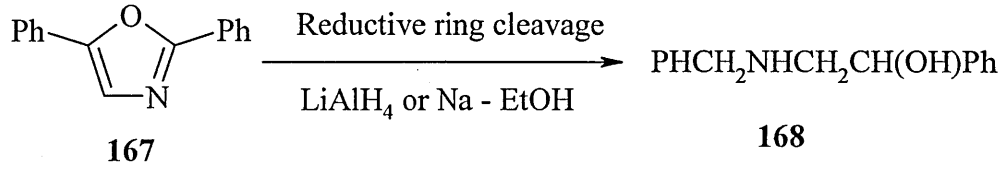
Scheme 75

Acid-induced ring cleavage initiates the reaction by the formation of the oxazolium salts which react much faster with nucleophiles. Reaction of 2,4,5-triphenyloxazole with dilute acid forms the oxazolium salt **166** which decomposes to yield benzaldehyde, benzoic acid, and ammonium chloride (scheme 76).



Scheme 76

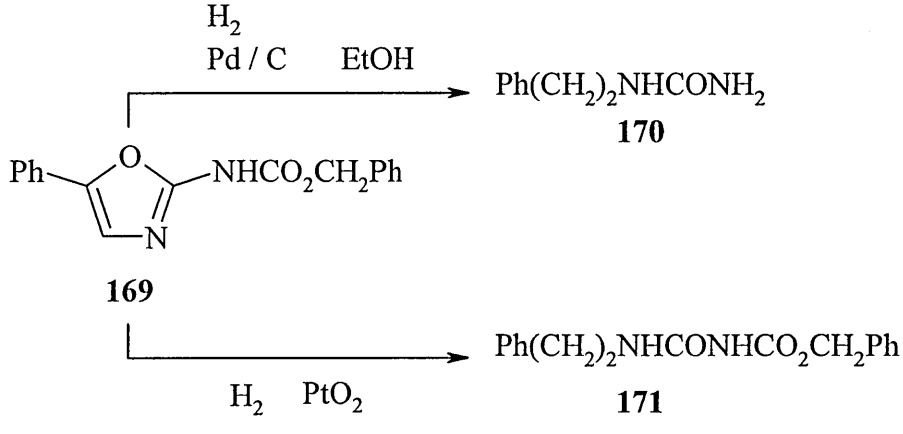
Oxazoles are stable towards a range of reductive conditions, but sodium in ethanol will convert oxazole to oxazolidines which may be reduced further to acyclic products[165]. A range of reductive ring cleavages is known; 2,5-diphenyloxazole **167** is cleaved by lithium aluminium hydride[171] or sodium in ethanol[165] to give 2-benzylamino-1-phenylethanol **168** (scheme 77).



167 **168**

Scheme 77

When benzyl 5-phenyl-2-oxazolecarbamate **169** was subjected to catalytic reduction in the presence of Pd/C in ethanol, phenethylurea **170** was isolated[169]. Catalytic hydrogenation of **169** using a PtO_2 catalyst gives 1-carbobenzyloxy-3-phenethylurea **171**, whereas Raney nickel was ineffective[169] (scheme 78). Reductive ring cleavage reactions of oxazoles can be inhibited by base; however, in the presence of acid the reaction is enhanced.



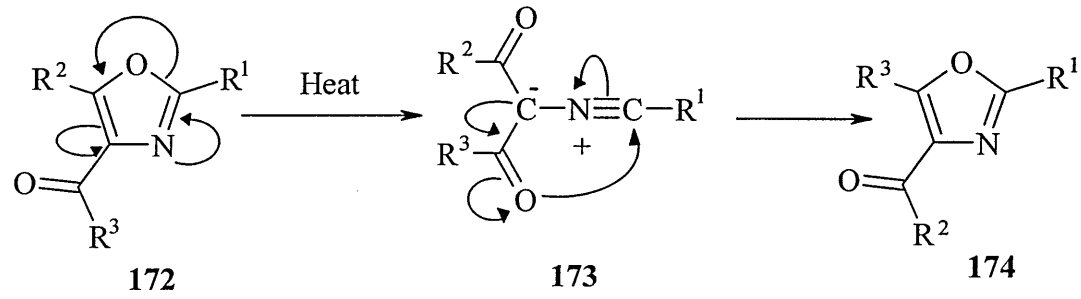
170

169

171

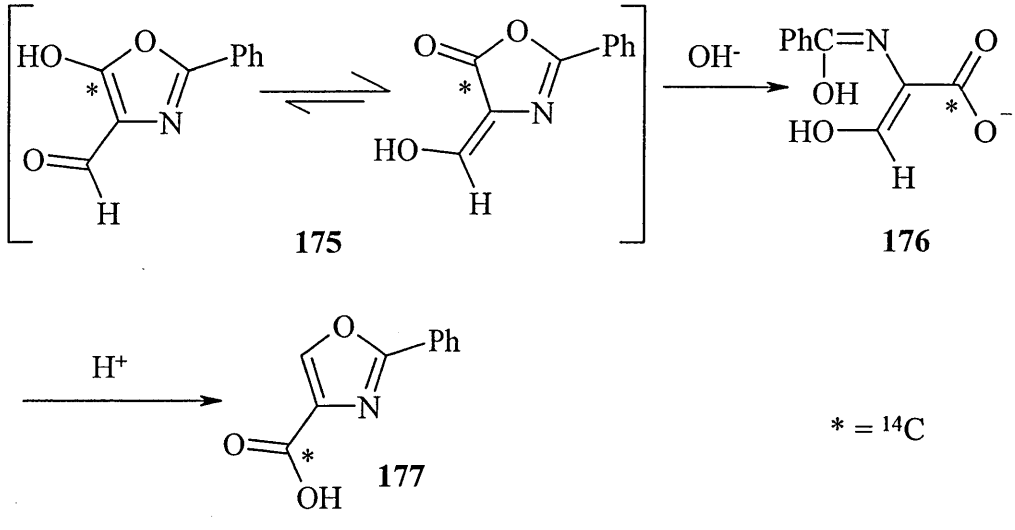
Scheme 78

The thermal rearrangement of 4-carbonyl substituted oxazoles is known as the Cornforth rearrangement[165]. When oxazole **172** is thermalised, the isomeric product **174** is obtained. The mechanism proposed[165] for the thermal isomerisation involves ring opening of the oxazole to the nitrile ylide intermediate **173** with subsequent ring closure to produce the rearranged oxazole **174** (scheme 79).



Scheme 79

The isotopic labelling experiments of base-induced isomerisation of 4-hydroxymethylene-5-oxazolones or their potassium salts **175** to oxazole-4-carboxylic acids **177** revealed the initial step of this base-induced rearrangement[172]. When the carbonyl carbon of the oxazolone ring was labelled with ^{14}C and the compound treated with base, the rearranged oxazole-4-carboxylic acid was labelled only at the carboxyl carbon atom. Thus, the initial attack of the hydroxide ion at the 2-position of the oxazolone ring with subsequent ring opening affords **176**, and ring closure yields the ^{14}C-labelled acid **177** (scheme 80).

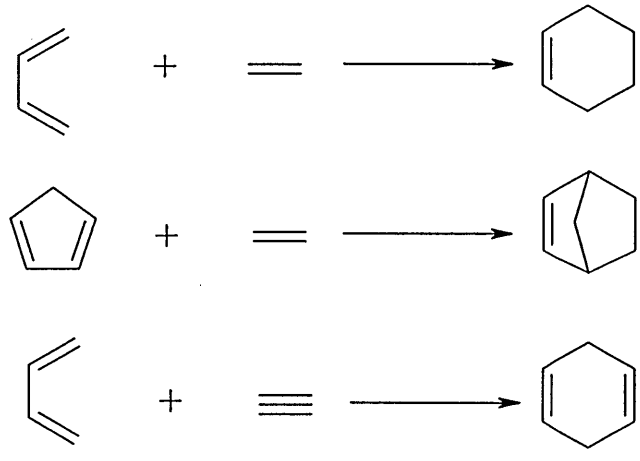


Scheme 80

1.5.2.3. Diels-Alder reactions.

Oxazoles undergo a variety of reactions mentioned above but one of the most important is the Diels-Alder reaction. Usually an electron-releasing substituent will facilitate the reaction with dienophiles. There are several variants of the reaction but the key step is the cycloaddition of an activated olefinic dienophile to an oxazole.

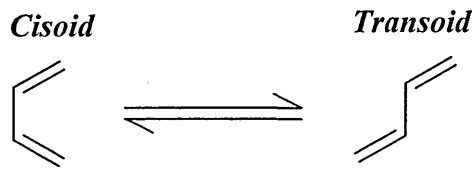
The Diels-Alder reaction is a cycloaddition reaction of a conjugated diene with a double or triple bond (the dienophile). In the classic Diels-Alder reaction a 1,3-diene reacts with a dienophile to form an adduct with a six-membered aromatic ring (scheme 81). The reaction proceeds by the formation of two σ-bonds and one π-bond at the expense of three π-bonds in the starting materials[173]. In general, the reaction takes place simply by mixing the components at room temperature or by gently warming in a suitable solvent. The Diels-Alder reaction is reversible, and many adducts dissociate into their components at quite low temperature. Heating is disadvantageous in these cases, however, using an excess of one of the components or a solvent from which the adduct separates readily will enhance the forward reaction and give better yields. Lewis acid catalysts are known to accelerate many Diels-Alder reactions, and there are a few instances where high pressures have been used to facilitate reactions which otherwise take place only slowly or not at all at room temperature[174,175].



Diene **Dienophile** **Adduct**

Scheme 81

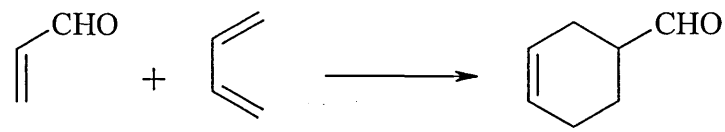
The importance of the Diels-Alder reaction is due to its versatility and its remarkable stereoselectivity. Many different types of ring structure can be developed by varying the nature of the diene and the dienophlie. In the majority of cases all six atoms involved in forming the new ring are carbon atoms, however, ring closure may also take place at atoms other than carbon to give heterocyclic compounds. Many dienes can exist in a *cisoid* and a *transoid* conformation (scheme 82), and it is only the *cisoid* form which can undergo addition. If the diene does not have, or cannot adopt a *cisoid* conformation no reaction occurs.



Scheme 82

1.5.2.4. Dienophiles.

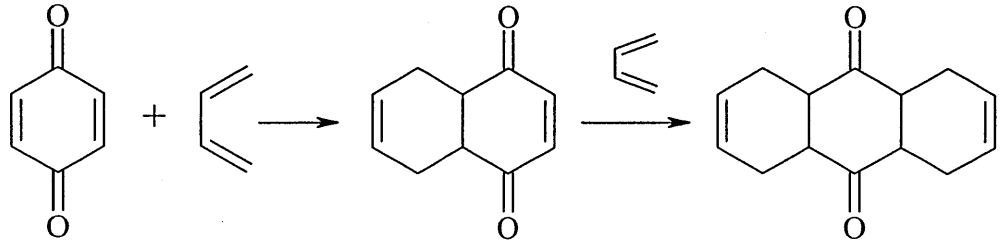
Dienophiles participate in the Diels-Alder reaction may be derivatives of acetylene (C_2H_2), ethylene (C_2H_4) or reagents in which one or both of the reacting atoms is a heteroatom. The reactivity of the dienophiles depends on its structure. In general, the greater the number of electron-attracting substituents on the double or triple bond the more reactive the dienophile is. Hence, the majority of the reactions involve an electron-rich diene and an electron-deficient dienophile. Dienophiles that contain one or more of CO, CO_2R, CN, or NO_2 groups in conjugation with a double or triple bond react readily with dienes. The most widely used dienophiles are probably the α,β-unsaturated carbonyl compounds, such as acrolein (propenal), acrylic acid and its esters, maleic acid and its anhydride, and numerous derivatives of 2-cyclohexenone. For instance, acrolein **178** reacts rapidly with butadiene **179** in benzene solution at 0 °C to produce tetrahydrobenzaldehyde **180** in quantitative yield (scheme 83).



178 **179** **180**

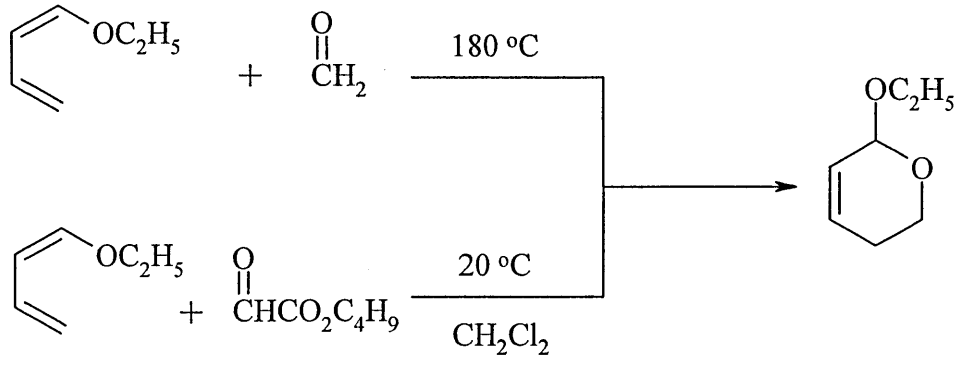
Scheme 83

The quinones are another important class of dienophiles of the α,β-unsaturated carbonyl class. *p*-Benzoquinone reacts readily with butadiene at room temperature to give the mono-adduct, tetrahydronapthaquinone, in high yield. However, under more vigorous conditions a bis-adduct is obtained which can be converted into anthraquinone by oxidation of an alkaline solution with atmospheric oxygen [176] (scheme 84).



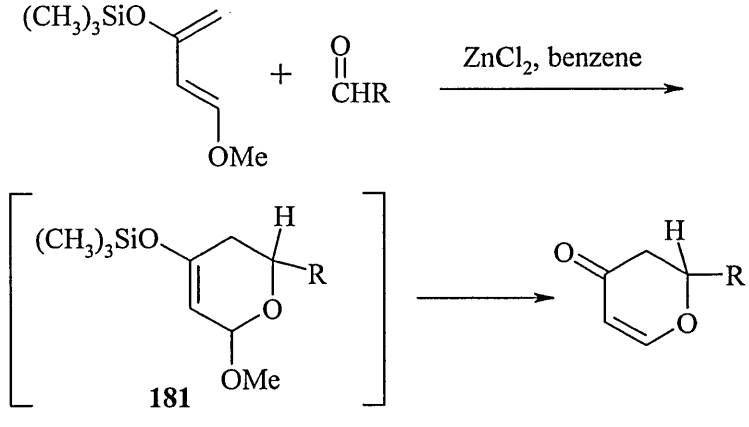
Scheme 84

The reaction of the carbonyl groups in aldehydes and ketones with dienes has been used to prepare derivatives of 5,6-dihydropyran. Formaldehyde reacts only slowly but reactivity increases with reactive carbonyl compounds such as esters of glyoxalic acid[177] (scheme 85).



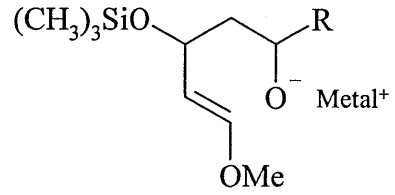
Scheme 85

The scope of this reaction is extended when zinc chloride or boron trifluoride etherate is present as catalyst. Under these conditions, oxygenated butadiene derivatives react readily with a wide range of aldehydes producing dihydro-γ-pyrones[178] (scheme 86). Zinc chloride catalysed reactions are cycloadditions and proceed through 1:1 cyclo-adducts such as **181**.



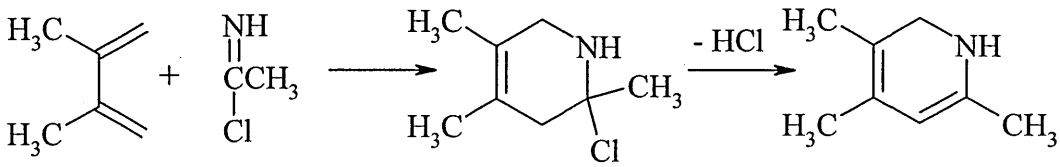
Scheme 86

On the other hand, the boron trifluoride catalysed reactions appear to proceed to produce an open-chain aldol-like product[178] **182**.



182

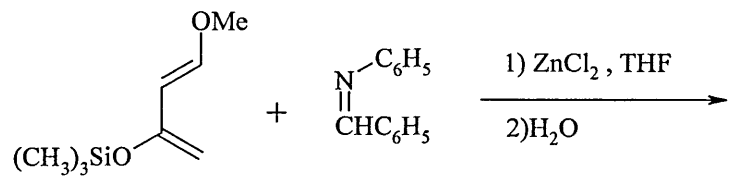
Another useful group of heterodienophiles are the imines, which contain the group –C=N-. The reaction of 2,3-dimethylbutadiene **183** and iminochloride **184** produces 3,4,6-trimethyl-1,2-dihydropyridine by elimination of hydrogen chloride from the initial adduct[179] (scheme 87).

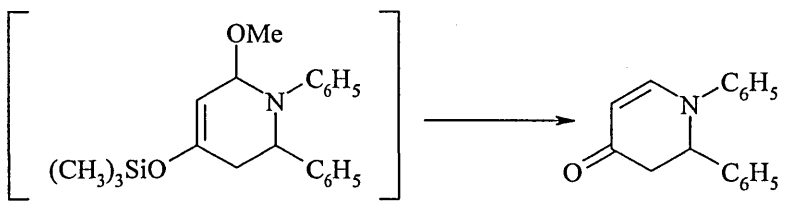


183 **184**

Scheme 87

It was assumed that only 'activated' imines in which either the nitrogen or carbon atom of the imine linkage, or both, carried an electron-withdrawing group, would undergo the intermolecular Diels-Alder reaction readily. However, 'ordinary' Schiff's bases react readily with butadiene derivatives in the presence of zinc chloride to form 2,3-dihydro-4-pyridones in good yield[180] (scheme 88).

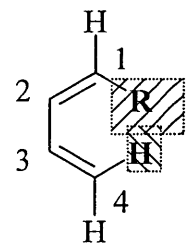




Scheme 88

1.5.2.5. Dienes.

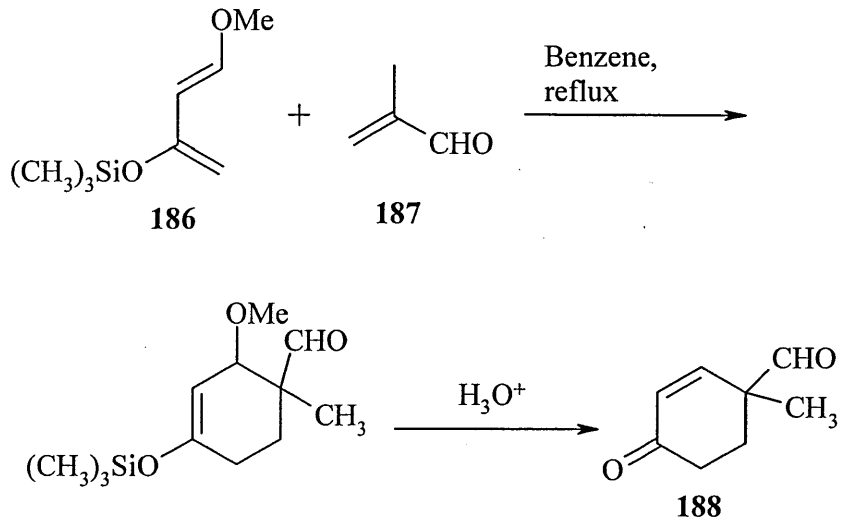
As mentioned earlier, the diene component must have or must be able to adopt the *cisoid* conformation before it can participate in Diels-Alder reactions with dienophiles. Dienes, which satisfy this condition, undergo the reaction more or less easily depending on their structure. Acyclic conjugated dienes react readily forming adducts in quantitative yield. Substituents in the diene can influence the rate of cycloaddition both through their electronic nature and by a steric effect on the conformational equilibrium. Electron-donating substituents (e.g. –Me, -OMe, $-NMe_2$) in the diene as well as electron-attracting substituents in the dienophile often increase the rate of the reaction. Bulky substituents that prevent the diene from adopting the *cisoid* conformation can hinder the reaction. For instance, 2-methyl-, 2,3-dimethyl-, and 2-t-butylbutadiene react normally with maleic anhydride, whereas the 2,3-diphenyl compound is less reactive and the 2,3-di-t-butylbutadiene is completely unreactive. The 2,3-di-t-butylbutadiene is prevented from attaining the necessary planar *cisoid* conformation by the steric effects of the two bulky substituents. Conversely, the substituents in 1,3-di-t-butylbutadiene do not interfere with each other even in the *cisoid* form, hence reacts readily with maleic anhydride[173]. Also, the *Z*-alkyl or aryl substituents in the 1-position of the diene **185** will reduce its reactivity by sterically hindering formation of the *cisoid* conformation through non-bonded interaction with the hydrogen atom at C-4. Thus, an *E*-substituted 1,3-butadiene will react with dienophiles much more readily than the *Z*-isomer.



R = alkyl or aryl

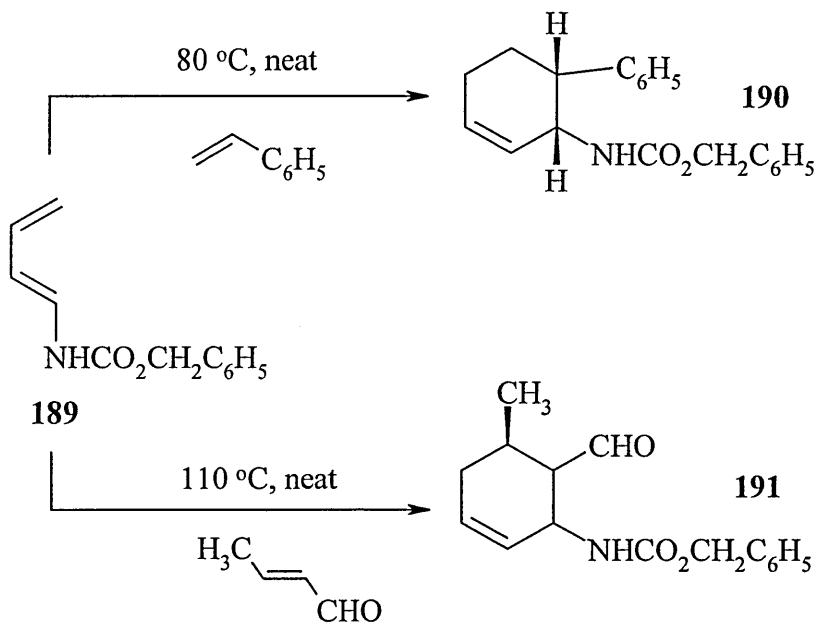
185

Derivatives of hydroxybutadienes, such as 1-methoxy-3-trimethylsily-oxybutadiene **186** (Danishefsky diene) have been employed in several syntheses of natural products[181]. Thus, the diene **186** reacts with 2-methylpropenal **187** to afford 4-formyl-4-methylcyclohexenone **188** after acid hydrolysis of the initial adduct (scheme 89)[182].



Scheme 89

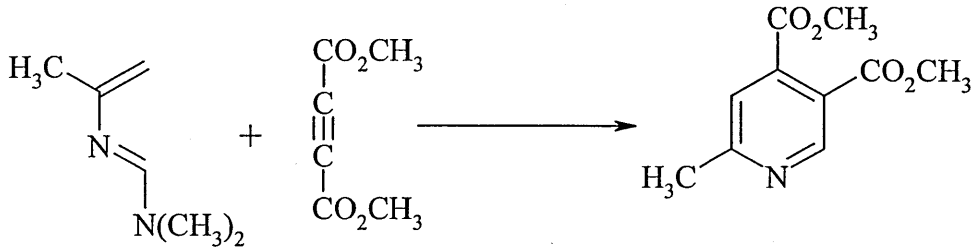
Heterosubstituted dienes such as the 1- and 2-acylaminobutadiene react readily with dienophiles to give substituted amino-cyclohexanes. The acylamino group is a powerful directing group and most reactions proceed with high regio- and stereo-selectivity. Hence, styrene reacted with diene **189** gave the 1,2 (*'ortho'*) addition product **190** almost exclusively, and with *trans*-crotonaldehyde the adduct **191** was obtained[183] (scheme 90).



Scheme 90

Heterodienes, in which one or more of the atoms of the conjugated diene is a heteroatom such as nitrogen, have provided very convenient access to some six-membered nitrogen heterocycles. Substituted 1-dimethylamino-2-azabutadienes **192**

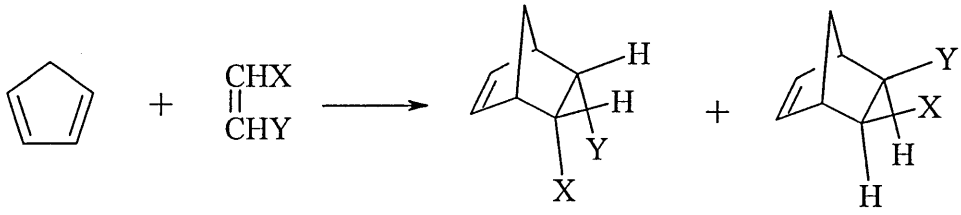
react with dienophiles **193** with loss of dimethylamine to give pyridines or dihydropyridines[184] (scheme 91).



192 **193**

Scheme 91

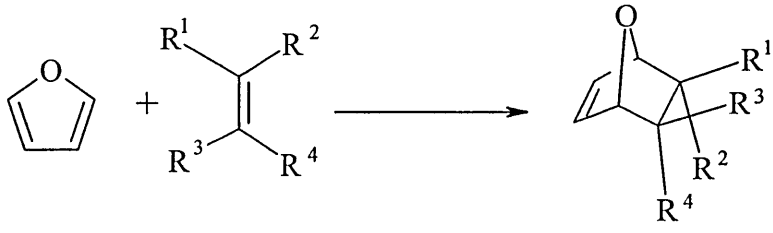
Cyclopentadiene, in which the double bonds are constrained in a planar *cisoid* conformation, reacts easily with a variety of dienophiles to form bridged compounds of the bicyclo[2,2,1]heptene series (scheme 92). The reaction of cyclopentadiene with mono- and *cis*-disubstituted ethenes can give rise to two stereochemically distinct products, the *endo*- and *exo*-bicyclo[2,2,1]heptene derivatives, however, the *endo* isomer always predominates in practice.



Endo *Exo*

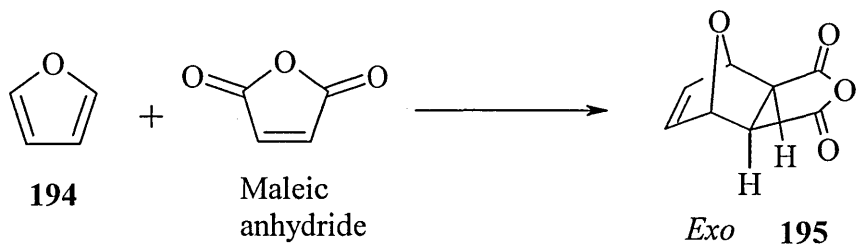
Scheme 92

Furan derivatives and related families, such as oxazoles, react with ethylenic and acetylenic dienophiles to form bicyclic compounds with an oxygen bridge (scheme 93). Usually adducts obtained from furans are thermally labile and dissociate readily into their components on warming.



Scheme 93

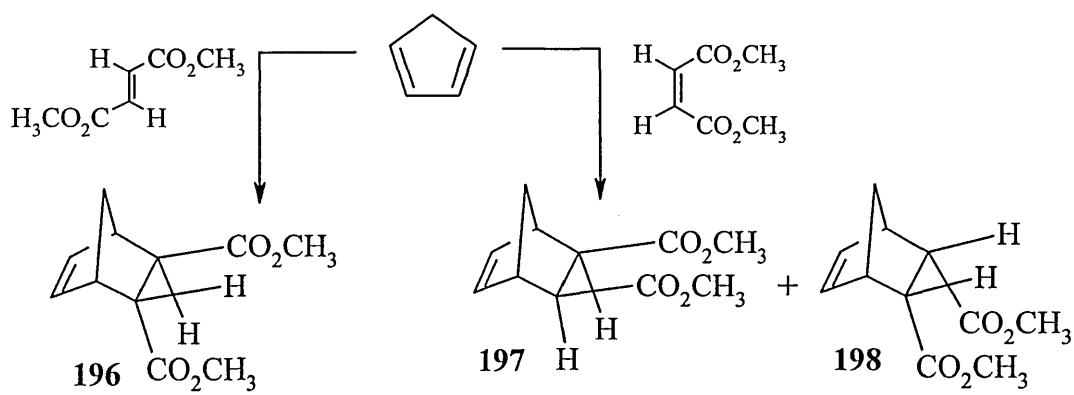
Furan **194** and maleic anhydride give an adduct with *exo* structure **195**, apparently, disobeying the rule that the *endo* isomer predominates (scheme 94). The reason is shown by the observation that the *endo* adduct formed from maleimide and furan at 20 °C dissociates at temperatures above 20 °C, allowing conversion of the *endo* adduct formed in the kinetically controlled reaction into the thermodynamically more stable *exo* isomer[173]. In the case with the maleic anhydride adduct, equilibration takes place below room temperature so that the *endo* adduct produced under kinetic control is not observed.



Scheme 94

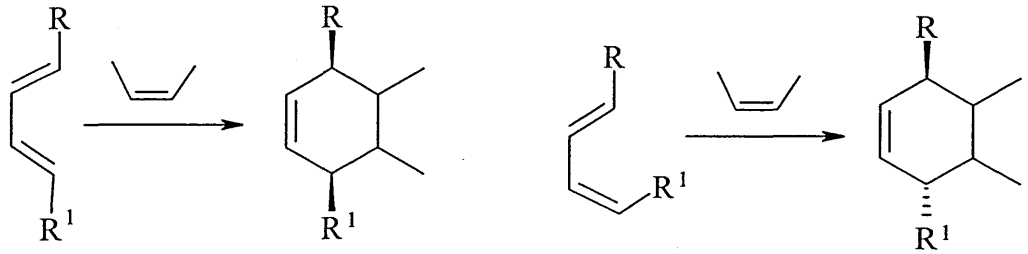
1.5.2.6. Stereochemistry of the Diels-Alder reactions.

The adduct stereochemistry obtained in many Diels-Alder reactions is based on two rules. The relative stereochemistry of substituents in both the dienophile and the diene is retained in the formed adduct on the basis of the '*cis* principle', also known as the *syn* addition[185]. A dienophile with *trans* substituents will give an adduct in which the *trans* configuration of the substituents is retained. With a *cis* disubstituted dienophile the formed adduct will have substituents that are *cis* to each other. For instance, in the reaction of cyclopentadiene with dimethyl fumarate the *trans* configuration of the ester groups is retained in the adduct **196**, while with diemethyl maleate the *cis* adduct **197** and **198** are formed (scheme 95).



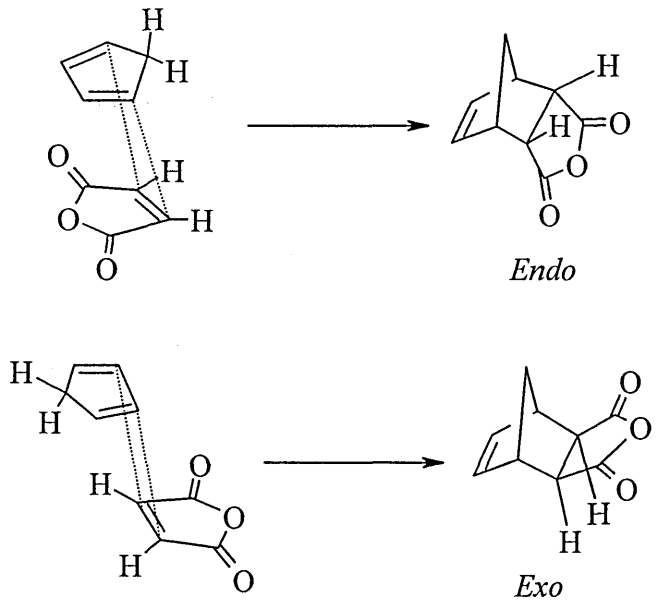
Scheme 95

Similarly, a *trans,trans*-1,4-disubstituted diene will give an adduct in which the 1- and 4-substituents are *cis* to each other, and a *cis,trans*-1,4-disubstituted diene forms an adduct with *trans* substituents (scheme 96).



Scheme 96

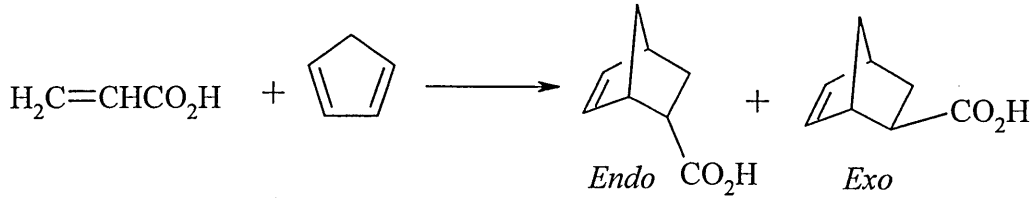
According to Alder's *endo* addition rule, in a diene addition reaction the two components arrange themselves in parallel planes, and the most stable transition state arises from the orientation in which there is 'maximum accumulation of double bond'[173,185]. The π-bonds of the activating groups in the dienophile also take part in the addition. The rule appears to be strictly applicable only to the addition of cyclic dienophiles to cyclic dienes. Thus, in the addition of maleic anhydride to cyclopentadiene, the *endo* product, formed from the orientation with maximum accumulation of double bonds, is produced almost exclusively and the *exo* product in a yield of less than 1.5 % (scheme 97).



Scheme 97

In addition of open-chain dienophiles to cyclic dienes, the *endo* rule is not always obeyed and the composition of the mixture obtained may depend on the precise

structure of the dienophile and on the reaction conditions. Thus, reaction of acyclic acid to cyclopentadiene produced *endo* and *exo* products in the ratio 75:25 (scheme 98).

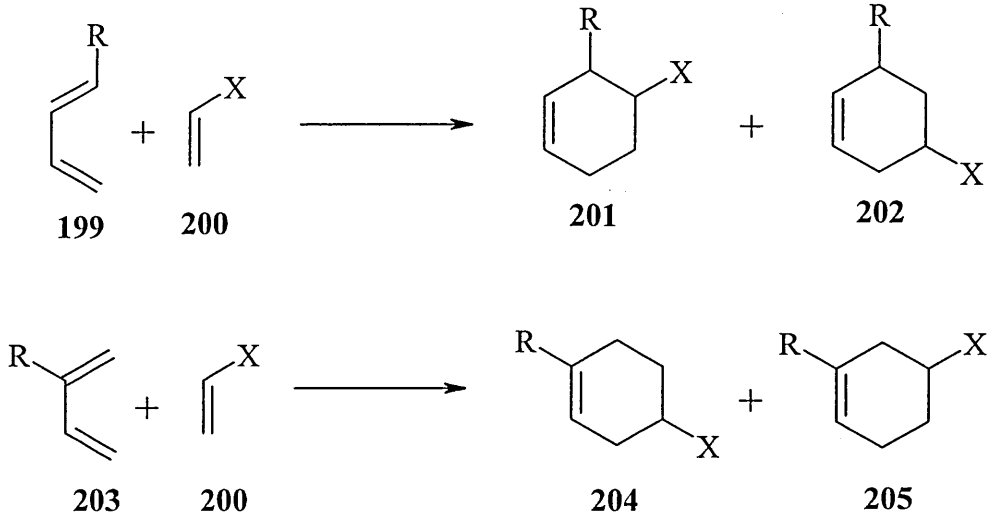


Scheme 98

In reactions of open-chain dienes and open-chain dienophiles the *endo* adducts are the main products at moderate temperature, but in some instances the proportion of the *exo* isomer increases with the rise of temperature.

1.5.2.7. Regiochemistry of the Diels-Alder reactions.

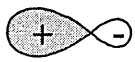
From reaction of an asymmetrically substituted dienes **199** and **203** with dienophile **200**, different regioisomeric products **201**, **202** and **204**, **205** can be formed (scheme 99).



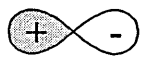
Scheme 99

The so-called '*ortho*' **201** and the '*para*' **204** isomers are formed preferentially. The observed regioselectivity can be explained by taking into account the frontier orbital coefficient of the reactant[186].

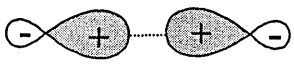
In a typical alkene, the σ and π orbitals form the bond. The molecular orbitals, σ-orbitals (sp^2 hybridised for alkenes, **206**) and π orbitals **207**, represent the electronic interactions. These orbitals take on a directional character (represented by + and -) when they are in close proximity to another orbital. When two sp^2 hybridised orbitals come together, orbitals of the same sign can be directed towards each other, as in **208**, or orbitals of opposite sign can be directed toward each other, as in **209**. In **208**, the electron density between the two nuclei is maximised (electronic attraction for the positive nucleus), but in **209** it is minimised. Since a strong covalent bond (mutual sharing of electron density between nuclei) is associated with significant electron density between the nuclei, **208** represents a bonding interaction and **209** an antibonding interaction.



206



207



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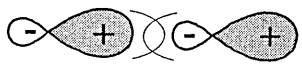
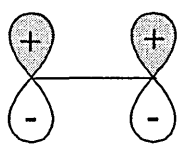
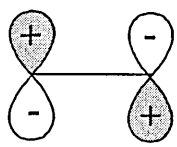


209

Similarly, the overlap of π orbitals to form a π bond with the same sign representing the maximum bonding interaction **210** (a bonding molecular orbital), and **211** represents the antibonding molecular orbital. As with a σ bond, the net energy of the bonding interaction in a π bond is lower than that of the antibonding interaction. An antibonding molecular orbital is higher in energy (less stable) than a bonding one.



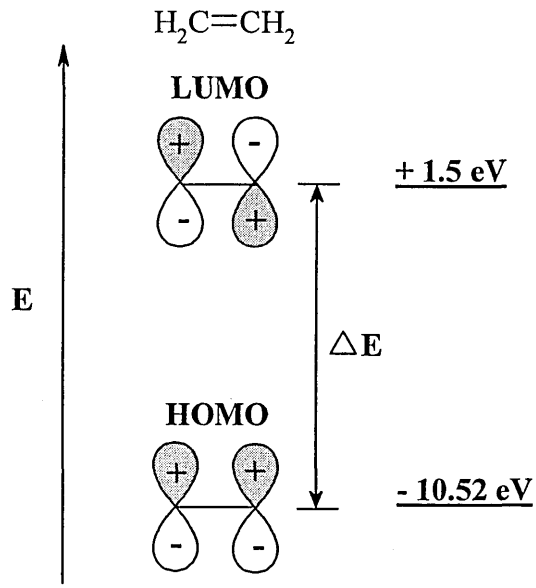
210



211

The difference in energy between the two π molecular orbitals (**210** and **211**) can be represented in terms of their relative energies[187]. The lowest energy orbital containing the bonding electrons is called the highest occupied molecular orbital (HOMO) and the ionisation potential (IP) of the molecule is a reasonable measure of the energy of this orbital[188]. In terms of chemical reactivity, the HOMO is the orbital which donates electrons. The higher energy orbital does not contain electrons, but is the next available energy level if electron is accepted. The energy which describes accepting electrons is the electron affinity (EA), and this orbital is called the lowest

unoccupied molecular orbital (LUMO). The LUMO accept electrons from the HOMO of another π bond and the difference in energy between the LUMO and HOMO ($\Delta E = E_{LUMO} - E_{HOMO}$) is determined by the difference of the EA and the IP. For ethene, the energy of HOMO is -10.52 eV, the LUMO is +1.5 eV therefore the ΔE is 12.02 eV (scheme 100).

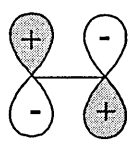
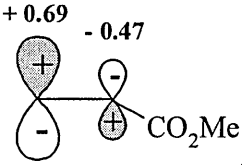
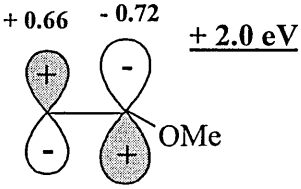
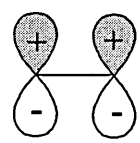
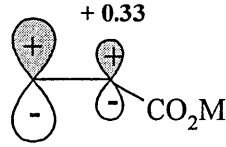
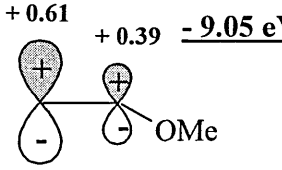


Scheme 100

The reactive HOMO and LUMO of ethene (or of any other molecule) are referred to as frontier molecular orbitals (FMO)[189]. The HOMO was believed to deliver electrons to the LUMO of a reactive centre, which could accept the charge. FMO theory has now been shown to be "a first approximation to a perturbation treatment of chemical reactivity"[190]. Perturbation theory[191] treats the molecular orbital of two interacting components as a perturbation of the product of their individual orbitals[192]. The theory fails to explain large perturbations and does not predict the transition state, but gives "an estimation of the *slope* of an early part of the path along the reaction coordinate leading to transition state"[192]. The transition state energies can be described as reactant-like or product-like. The Hammond postulate states, "The transition states for exothermic reactions are reactant-like, and for endothermic reactions are product-like"[193]. Frontier orbital effects are therefore particularly important in exothermic reactions[194].

In **212** and **213**, the magnitude of each orbital, called the orbital coefficient, is shown. The + and – signs correlate with the + (darkened) or – (undarkened) symmetry of the orbital[188]. When two π systems differ in their substitution pattern,

there will be differences in the magnitude of orbital densities ('larger' or 'smaller' orbitals). The orbital coefficients of a symmetrical molecule such as ethene are identical in magnitude and sign for the HOMO. The LUMO coefficients are equal in magnitude but opposite in sign, also a result of this symmetry, and this leads to one node in **212** and no node in **213** (a node is a point of zero electron density, such as a nucleus). Low energy orbitals are associated with a minimal number of nodes, and higher energy orbitals with increasing numbers of nodes. If an electron-withdrawing group is attached to ethene, as in methyl acrylate, electron density is distorted away from the π bond of the alkene, towards the carbonyl. This will influence the energies of the HOMO and LUMO as well as the magnitude of the orbital coefficients. The electron-withdrawing group diminishes the size (orbital coefficient) of the orbitals closest (proximal) to the $\delta+$ carbonyl carbon, leaving the most distant (distal) orbital with the largest coefficient. If an electron-releasing group is attached to ethene, as in methyl vinyl ether, the oxygen atom releases electrons toward the π bond, raising the energy of the orbitals relative to ethene (table 3).

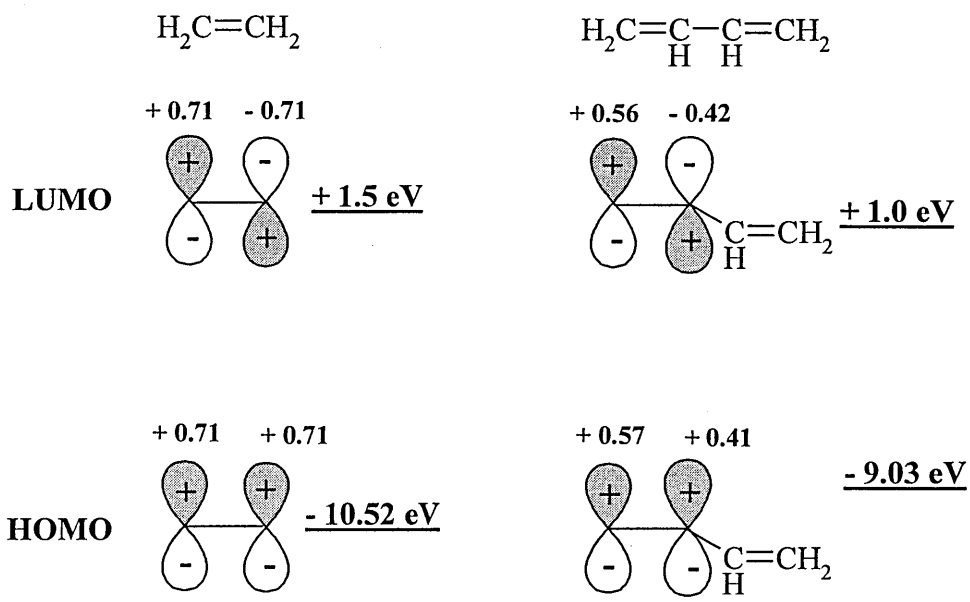
$H_2C{=}CH_2$	$H_2C{=}CHCO_2Me$ (electron-withdrawing)	$H_2C{=}CHOMe$ (electron-releasing)
+ 0.71 - 0.71  + 1.5 eV **LUMO 212**	+ 0.69 - 0.47  CO_2Me 0 eV	+ 0.66 - 0.72 + 2.0 eV  OMe
+ 0.71 + 0.71  - 10.52 eV **HOMO 213**	+ 0.43 + 0.33  - 10.72 eV CO_2Me	+ 0.61 + 0.39 - 9.05 eV  OMe

Changes in alkene HOMO and LUMO energies upon addition of substituents.

Table 3

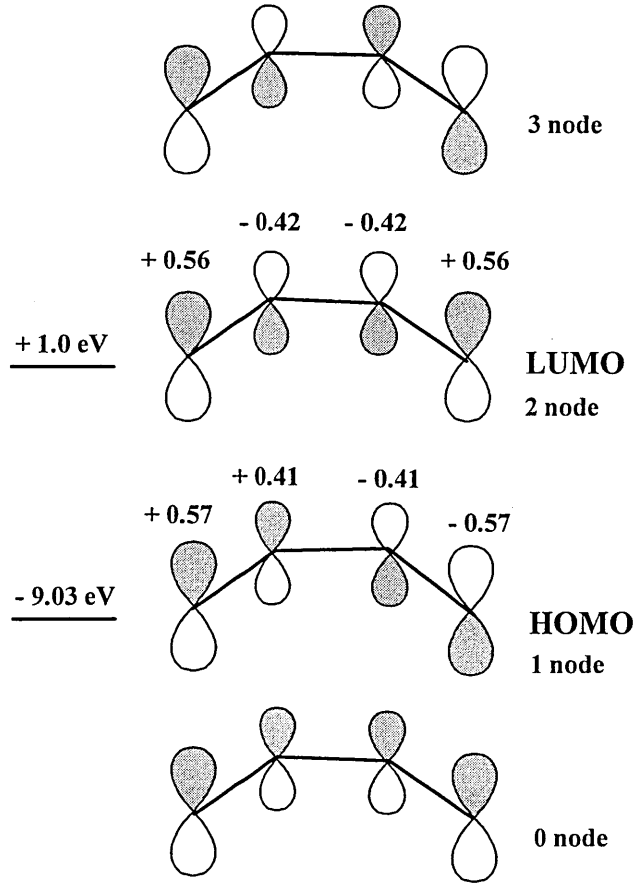
In 1,3-butadiene, where the conjugated substituent is another ethene moiety, the HOMO energy is -9.03 eV (with coefficient of 0.57 and 0.41) and the LUMO

energy is lowered to +1.0 eV (with coefficient of 0.56 and -0.42), relative to ethene[195] (scheme 101).



Scheme 101

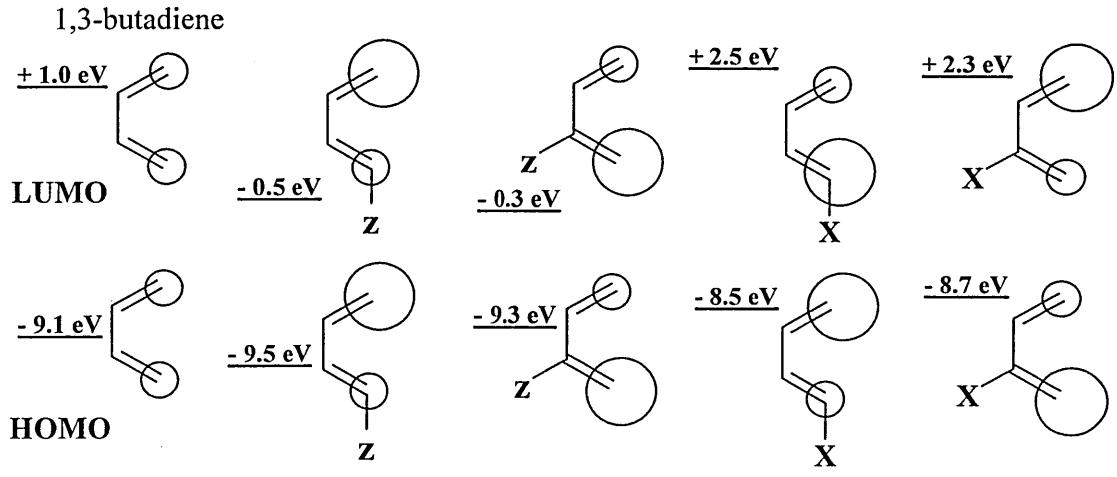
Although the HOMO-LUMO coefficients for all four atoms of the conjugated system must be considered, only those at the termini will interact with the alkene in a Diels-Alder reaction. The 1,3-butadiene is symmetrical but the coefficients will reflect the electron-withdrawing effect invoked in the distribution of electron density in the orbitals. The largest concentration of electron density occurs at the terminal carbons, and these are expected to be the major sites of reactivity. Since there are four π orbitals and four π electrons, there are four molecular orbitals, as shown in scheme 102. The lowest energy orbital has no nodes, the HOMO has one node, the LUMO has two nodes, and the highest energy orbital has three nodes. In scheme 102, the lowest two energy orbitals contain electrons, with the HOMO being the higher energy orbital. The HOMO will be more important for reactions than other orbitals, since it contains electrons which can be donated. Similarly, the LUMO is the lowest energy of unfilled orbitals and is more important for reactions, since it will preferentially accept electrons.



Orbital diagram for 1,3-butadiene

Scheme 102

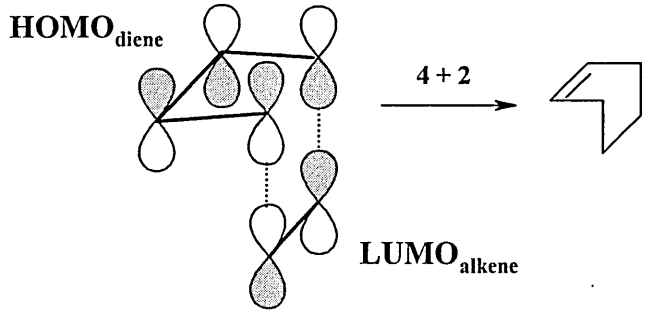
The effects on the frontier orbitals in diene with attached substituents are similar to those observed in alkenes. Electron-withdrawing groups will decrease the energy of the HOMO and LUMO relative to butadiene. Conversely, electron-releasing groups will increase the HOMO and LUMO energies. In scheme 103, the substituent can be attached to C-1 or C-2 of the diene, where the Z group is a generic electron-withdrawing group and the X group is a generic releasing group. The electron-withdrawing groups (Z) lower the HOMO and the LUMO. The C-1 substituent lowers the energy more than a C-2 substituent and the orbital coefficients are larger on the orbitals distal to the Z group for C-1 substituents, although they are larger on the proximal carbon for C-2 substituents. Electron-releasing groups (X) raise the energies of the HOMO and the LUMO. The orbital on the carbon distal to X is smallest for C-1 substituents and largest for C-2 substituents in the LUMO. In the HOMO the orbital on the distal carbon is largest for C-1 substituents and smallest for C-2 substituents[186].



HOMO and LUMO energies of electron rich and electron poor dienes, compared with 1,3-butadiene.

Scheme 103

Reactions that involve multiple π bonds can take many forms, but the number of π electrons in each substrate usually classifies the reaction. A single π bond can interact with another single π bond of an alkene and the reaction of two alkene is classified as a 2 π + 2 π reaction. When a diene reacts with an alkene there are six π electrons, therefore, known as a 4 π + 2 π reaction (scheme 104). The Diels-Alder reaction is recognised as the [4 + 2] cycloaddition.

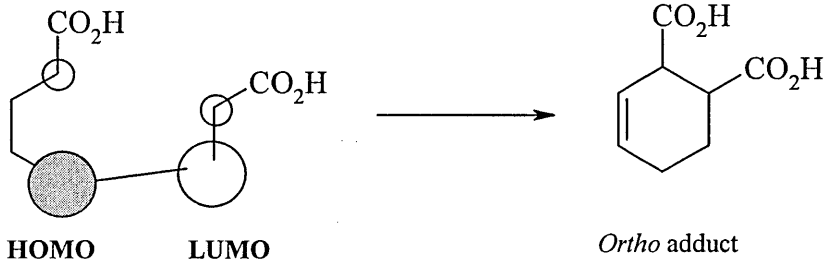


Scheme 104

Furthermore, reactions can be classified as 'allowed' or 'forbidden' reaction in correlation with the symmetry of the frontier orbitals of substrates[186]. In a reaction, maximum stability in the transition state is observed when the terminal carbons of the $HOMO_{diene}$ interact with the termini of the $LUMO_{alkene}$. These orbitals have the same symmetry, and such interaction is 'allowed' (scheme 104). If the orbitals have the opposite symmetry (and sign), the interaction is 'forbidden'[196]. However, a reaction

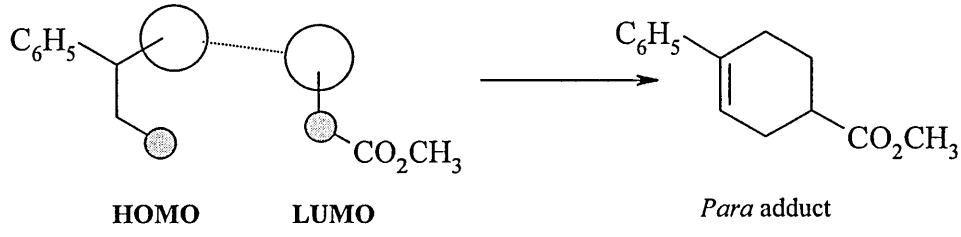
that is 'forbidden' may still take place if nothing easier is available. What is meant by 'forbidden' is that the interaction of the orbitals presents an energy barrier which the 'allowed' reactions do not have.

Therefore, in Diels-Alder reactions which involve an electron-deficient dienophile and an electron-rich diene, the main interaction is that between the highest occupied molecular orbital (HOMO) of the diene and the lowest unoccupied orbital (LUMO) of the dienophile. The smaller the energy differences between these orbitals and the better the overlap, the more readily the reaction occurs[197]. The orientation of the product obtained from an unsymmetrical diene and an unsymmetrical dienophile is governed largely by the atomic orbital coefficients at the termini of the conjugated systems concerned. The atoms with the larger termini coefficients on each add bond preferentially in the transition state, since this leads to better orbital overlap. This results to mainly the 1,2 ('*ortho*') adduct with 1-substituted butadienes and to the 1,4 ('*para*') adduct with 2-substituted butadienes. For the reaction of butadiene-1-carboxylic acid with acrylic acid the frontier orbitals are polarised, where the size of the circles is roughly proportional to the size of the coefficients. Note; shaded and empty circles represent lobes of opposite sign; an 'allowed' reaction involves overlap of lobes of the same sign (scheme 105).



Scheme 105

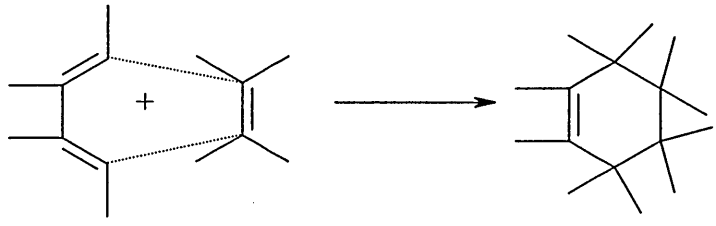
Similarly for the reaction of 2-phenylbutadiene and methyl acrylate, preferential formation of the '*para*' adduct is predicted (scheme 106).



Scheme 106

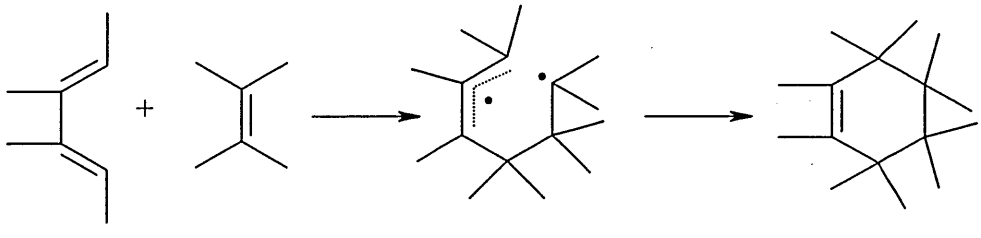
1.5.2.8. Mechanism of Diels-Alder reactions.

The rate-determining step in adduct formation is bimolecular, where dienophile and diene approach each other in parallel planes roughly orthogonal to the direction of the new bonds about to formed. Hence, formation of the two new σ-bonds take place by overlap of molecular π-orbitals in a direction corresponding to endwise overlap of atomic *p*-orbitals. For most Diels-Alder reactions a concerted addition in which both of the new single bonds are formed at the same time is generally accepted[173] (scheme 107).



Scheme 107

In some cases, reactions may proceed via diradical intermediate in a two step mechanism[185]. The first step is the formation of a single bond between atoms of the reactants which is rate controlling. The addition is then completed by formation of the second bond in a fast reaction (scheme 108).

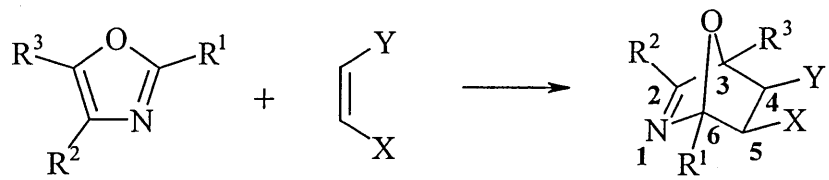


Scheme 108

The Diels-Alder reaction is of wide scope. The usefulness of the Diels-Alder reaction is based on its *syn*-stereospecificity, with respect to the dienophile as well as the diene, and its predictable regio- and *endo*-selectivities. Not all of the atoms involved in ring formation have to be carbon atoms. Thus, the hetero-Diels-Alder reaction involving one or more heteroatom centers can be used for the synthesis of six-membered heterocycles, such as substituted pyridines with oxazoles as the active dienophile.

1.5.2.9. Diels-Alder reaction of oxazoles with dienophiles.

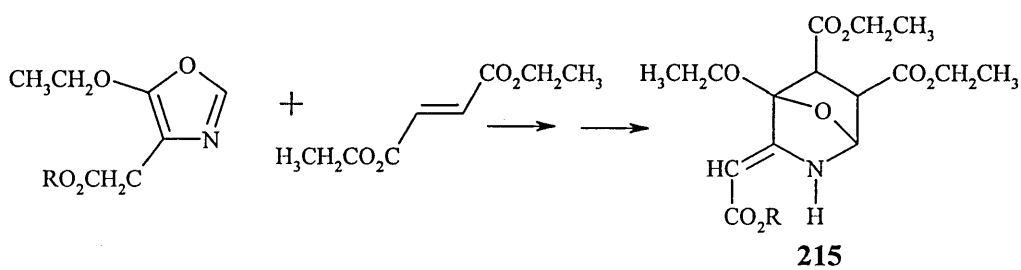
The Diels-Alder reaction of substituted oxazoles as diene with olefinic dienophiles to yield pyridine derivatives consists of two stages. The first stage results in the intermediate formation of bicyclic adduct **214** (scheme 109). Early studies assumed that the π-electron densities of the atoms, which participate in bonding in the transition state, governed the regioselectivity of the addition. However, consideration of the calculated π-charge densities does not always lead to the correct prediction of the regiochemistry of the adduct[157]. It was localisation energy calculation that provided the correct prediction of the orientation of oxazole-dienophile systems[198]. A general rule for predicting the orientation of the addition is that the more electronegative substituent on the dienophile (Y) occupies the 4-position in the adduct[199].



Substituted oxazole **214**

Scheme 109

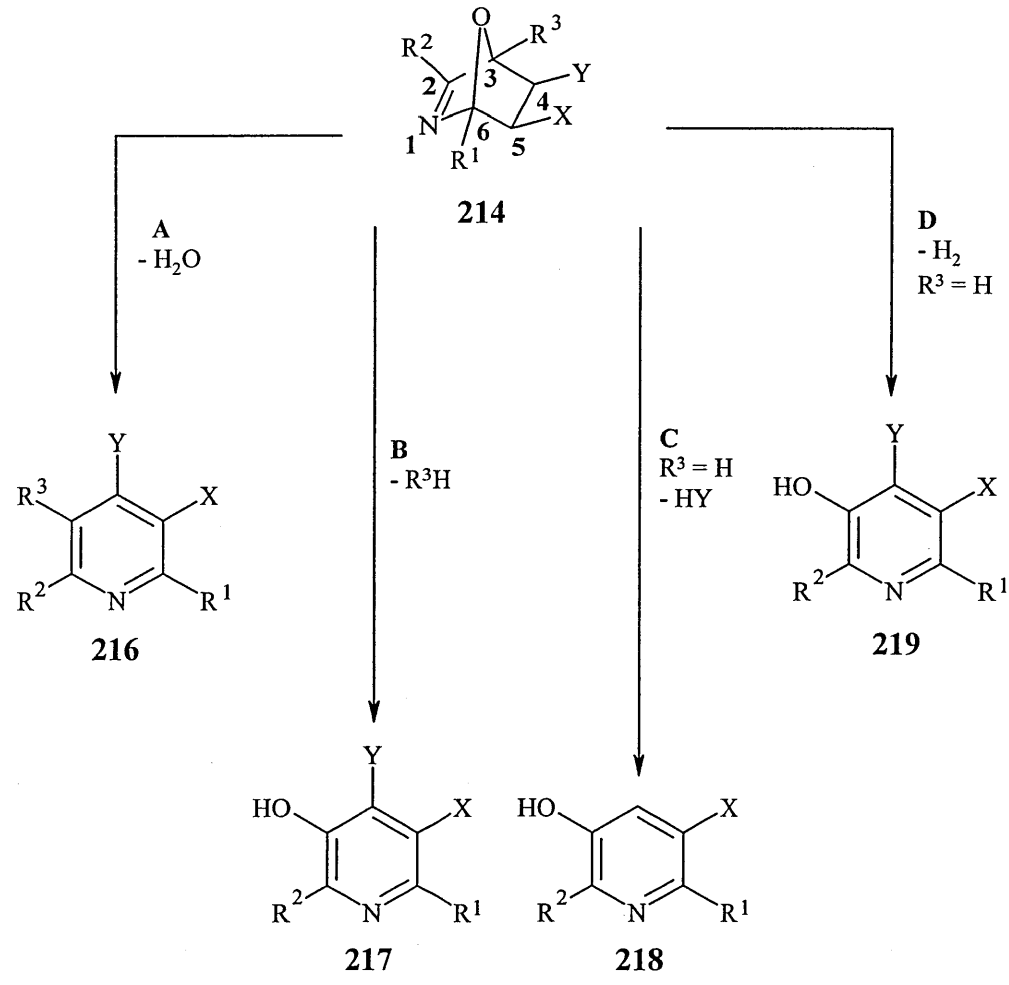
Normally, the bicyclic adducts **214** are not isolated from the reaction, however, the reaction of 5-ethoxyoxazole-4-acetic acid and its esters with diethyl fumarate did give adduct **215** which is stable enough to be isolated[157]. The conjugation of the exocyclic double bond with the carbonyl group of the ester made adduct **215** more stable than **214** (scheme 110).



215

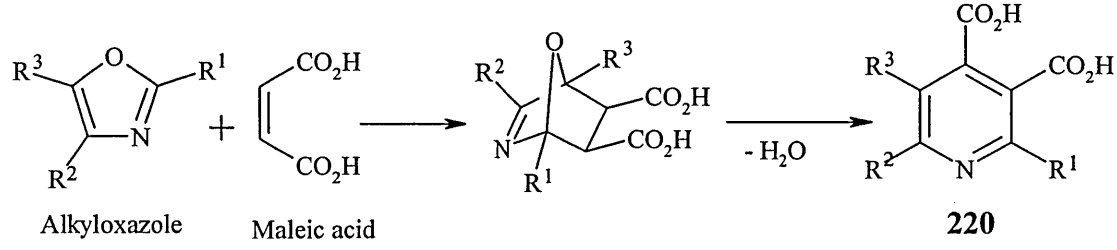
Scheme 110

The bicyclic adducts **214** normally undergo aromatisation, particularly in acidic conditions. Depending on the structure of the starting materials either one of four pathways can occur (scheme 111).



Scheme 111

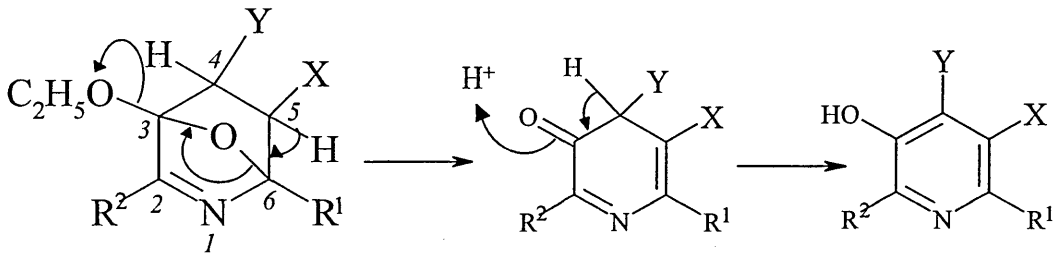
The aromatisation of the adduct **214** depends on the nature of the groups at the 3- and 4-positions (R^3 and Y). Pathway **A** is aromatisation with elimination of a water molecule and occurs when the substituents R^3 and Y are not good anionic leaving groups (e.g. alkyl). Alkyloxazoles react in this way with maleic anhydride and maleic acid to form alkyl-substituted cinchomeronic acids **220** (scheme 112)[200].



Scheme 112

If a substituent which is readily eliminated in the form of an anion is present in the 3-position (R^3 = OR, $OCO_2C_2H_5$, or CN) of the adduct **214**, then aromatisation takes

place via pathway **B** and the final product being substituted 3-hydroxypyridine **217**. The proposed mechanism for the aromatisation of the adducts begins with the dissociation of the C^6-O bond (scheme 113)[157]. Due to the σ-π conjugation with the C^2=N bond, the C^6-O bond is the one weakened to the greatest extent. Under the aromatisation conditions, dry hydrogen chloride in alcohol, the heterolysis is favoured by the protonation of the adduct both at the nitrogen and at the oxygen atom. As the cycloaddition and the aromatisation takes place in the absence of water, it is unlikely that the dissociation of the C^3-O bond with subsequent hydrolysis could occur.

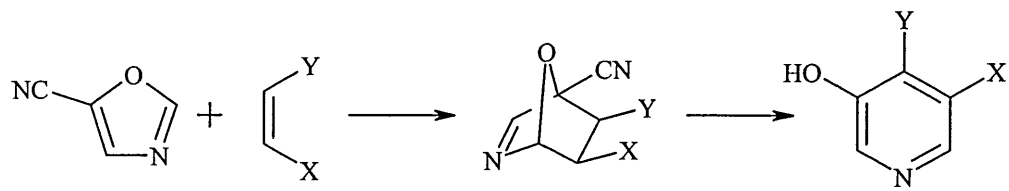


Adduct

217

Scheme 113

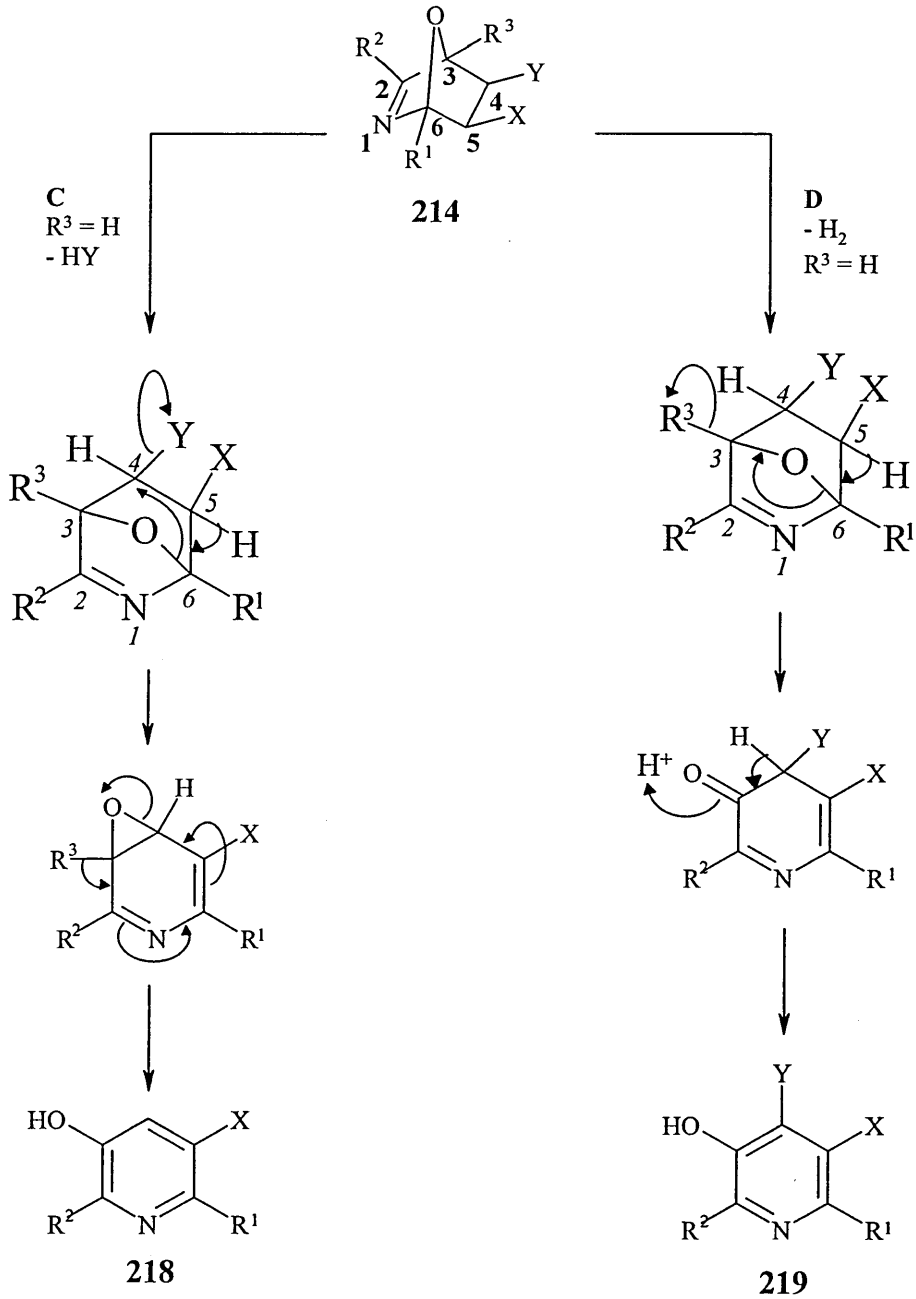
In addition, the interaction of 5-cyano-oxazoles with dienophiles formed the 3-hydroxypyrdines exclusively; therefore, the dissociation of the C^3-O bond is unlikely to occur[157] (scheme 114).



Scheme 114

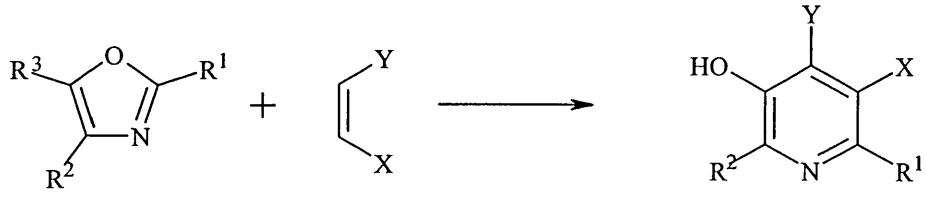
The aromatisation of adducts via pathways **C** and **D** is characteristic of the interaction of 5-unsubstituted oxazoles with dienophiles. If R^3 = H and Y is a good leaving group, then pathway **C** leading to **218** arises. If R^3 = H and Y is not a good leaving group, pathway **D**, oxidation of the adduct, can take place (scheme 115). Pathway **C** and **D** aromatisation of the adduct begins with the dissociation of the C^6-O bond of the oxygen bridge. In pathway **D**, R^3 is removed as a hydride ion, and since this type of process is energetically unfavourable, the product is usually obtained in insignificant amounts. However, if a hydride acceptor such as nitrobenzene or H_2O_2 is present in

the reaction mixture the yields of the corresponding pyridines **219** increased to 50 -60 %[200].



Scheme 115

The synthetic use of Diels-Alder condensation of oxazoles with dienophiles to form pyridines and especially of 5-alkoxyoxazoles, which react by pathway B to yield pyridoxine and pyridoxine derivatives, is well documented[157]. Various combinations of 5-alkoxyoxazoles with dienophiles, which undergo the Diels-Alder reaction to form the corresponding 3-hydroxypyridines **217**, are listed on table 4.



Substituted oxaozle

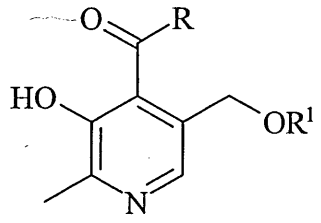
217

Substituents in oxazole			Dienophile		Pyridine product (217)				Ref.
R^1	R^2	R^3	X	Y	R^1	R^2	X	Y	
H	CH_3	OEt	CO_2Et	CO_2Et	H	CH_3	CO_2Et	CO_2Et	[201]
H	CH_3	OEt	CH_2OR	CH_2OR	H	CH_3	CH_2OR	CH_2OR	[201]
H	CH_3	OCO_2Et	CO_2Et	CO_2Et	H	CH_3	CO_2Et	CO_2Et	[202]
H	CH_3	OCO_2Et	CN	CN	H	CH_3	CN	CN	[202]
H	CH_2CO_2H	OEt	CH_2OH	CN	H	CH_3	CH_2OH	CN	[203]
H	CH_2CO_2Et	OEt	CH_2OH	CH_2OH	H	CH_3	CH_2OH	CH_2OH	[204]
H	CH_2CO_2Et	OEt	CN	CN	H	CH_3	CN	CN	[204]
H	CH_2CO_2Et	OEt	CO_2Et	CO_2Et	H	CH_2CO_2Et	CO_2Et	CO_2Et	[205]

Table 4

1.6. Aims.

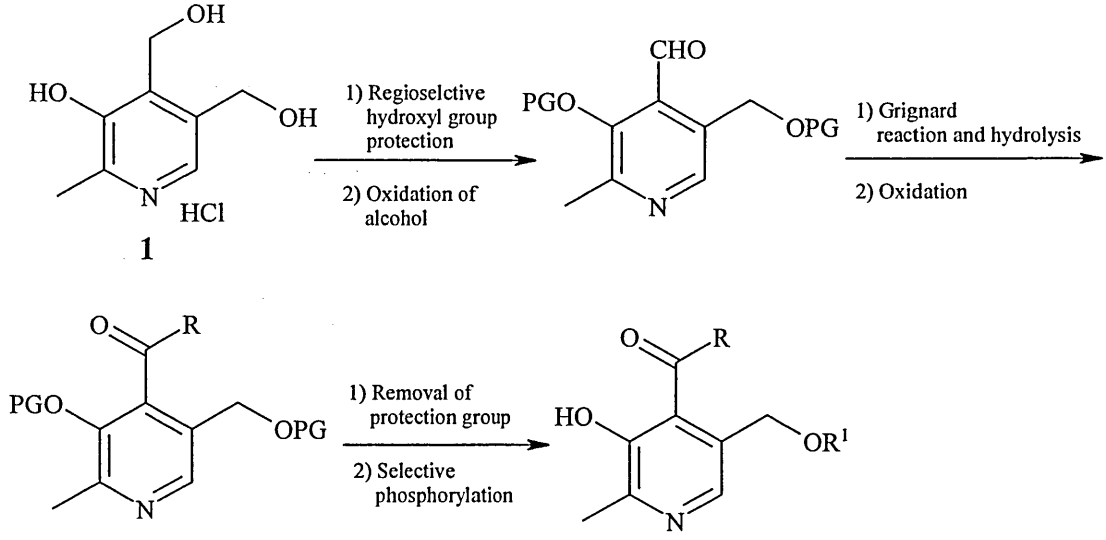
This study involves the design and preparation of pyridoxal 5'-phosphate analogues **220**. The analogues will be synthesised through the manipulation of commercially available vitamin B_6 and via the Diels-Alder cycloaddition of the substituted oxazoles with dienophiles.



R = CH_3, C_2H_5 or longer chain.
R^1 = H or PO_3H_2

220

Manipulation of readily available pyridoxine hydrochloride **1** begins by blocking the hydroxyl groups on the C-3 and C-5' position with protection groups and allowing the hydroxyl group at C-4' to be transformed into an aldehyde. The aldehyde derivative when subjected to a Grignard reaction and subsequent oxidation will give the desired ketone **221**. The removal of the protection groups and subsequent selective phosphorylation will achieve the phosphate form of analogues **220** (scheme 116).



1

R = CH_3, C_2H_5 or longer chain.

221

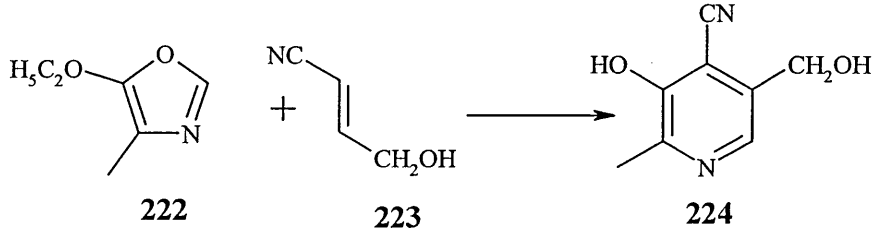
R = CH_3, C_2H_5 or longer chain.
R^1 = H or PO_3H_2

220

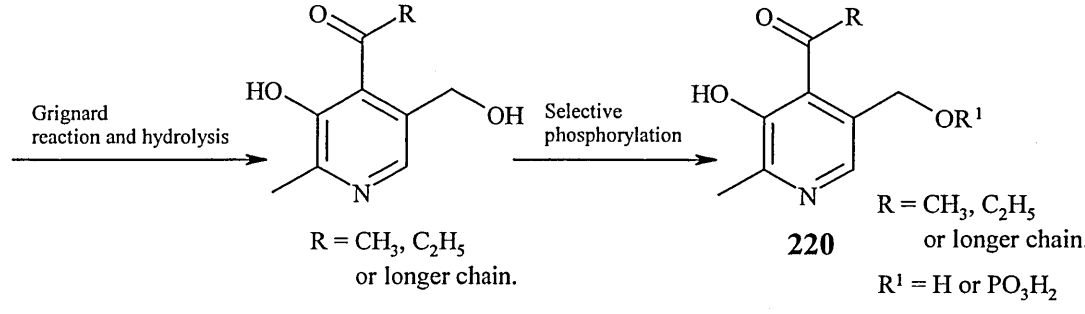
Scheme 116

Synthesis of the analogues **220** using the Diels-Alder reaction begins with the preparation of 4-methyl-5-ethoxyoxazoles **222** and the dienophiles, 4-hydroxybut-2-

enenitriles **223**. The oxazole **222** will interact with the dienophile **223** to yield the substituted pyridine **224**. The dienophile utilised in the Diels-Alder reaction should show a high degree of regioselectivity where the more electron withdrawing substituent of the dienophile ends up at the 4-position of the pyridine ring. The substituted pyridine **224** will then be subjected to modification at the 4-position via Grignard reaction. Subsequent phosphorylation will achieve the phosphate form of analogues **220** (scheme 117).



222 **223** **224**

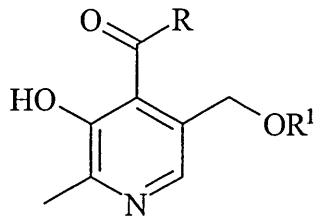


Scheme 117

The desired pyridoxal 5'-phosphate analogues will act as substrates to develop understanding of the interaction between the enzyme-substrate complexes in the enzymatic binding sites of pyrimidine biosynthesis in mammalian cells. The three enzymes, glutamine-dependent carbamoyl-phosphate synthetase (CPSase II), aspartate transcarbamoylase (ATCase) and dihydroorotase (DHOase), found in the multifunctional polypeptide CAD initiates pyrimidine biosynthesis. The key enzyme, CPSase II is regulated in the cell negatively by uridine triphosphate (UTP) and positively by 5-phosphoribosyl 1-pyrophosphate (PRPP). Earlier research[7,10] showed that UDP-pyridoxal and pyridoxal 5'-phosphate have similar activating activity on the CPSase II of the multifunctional polypeptide CAD. As biosynthesis of pyrimidine is essential for most growing cells, designing pyridoxal 5'-phosphate analogues and the feedback information about the binding sites in the mammalian multifunctional polypeptide CAD will be valuable for the development of antiproliferative agents.

2. Results and Discussion

The objectives of this study were to synthesise pyridoxal 5'-phosphate analogues **220** through the manipulation of commercially available pyridoxine and via the Diels-Alder cycloaddition of the substituted oxazoles with dienophiles.

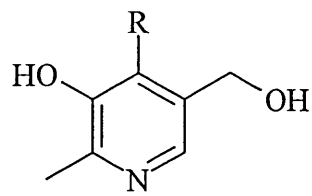


220

$R = CH_3$, C_2H_5 or longer chain.
$R^1 = H$ or PO_3H_2

2.1. Modification of pyridoxine.

Pyridoxine analogues obtained by modification of the 4-position have been of considerable interest in enzymatic and pharmacological studies[1,149]. Compounds such as 4-deoxypyridoxine (**7**, R = CH_3) are potent antagonists of vitamin B_6 in some mammalian systems[206], but in some tissues are subject to demethylation[207].

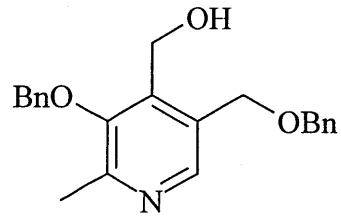


7 R = CH_3
225 R = CF_3
226 R = H
227 R = OH
228 R = CH=NHNHR

Replacement of the C-4-methyl H's in analogue **7** with F (**225**, R = CF_3) renders the compound less active in various systems[208], and replacement of the entire 4-side chain with H (**226**, R = H)[209] or with OH (**227**, R = OH)[210] considerably reduces inhibitory potency in *Saccharomyces carlsbergensis*. On the other hand, replacement of the aldehydic oxygen of pyridoxal with bulky nitrogenous groups, such as hydroximino, azino, and various hydrazone groups (**228**, R = CH=NHNHR), makes them powerful inhibitors of pyridoxal kinase *in vitro*[211]. Compounds of this type are also of some biological interest as inhibitors of human neoplastic cells *in vitro*[212] and retarders of tumour growth[213]. Some pyridoxine analogues that have the C-5-CH_2OH group unchanged are susceptible to phosphorylation catalysed by pyridoxal kinase[211], and

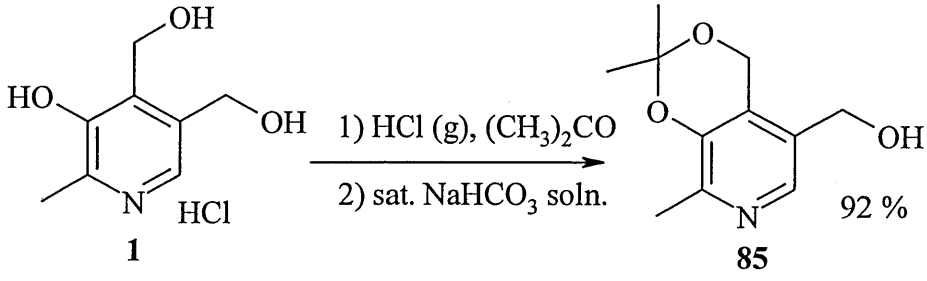
the phosphorylated analogues are capable of effective competition with the coenzyme pyridoxal 5'-phosphate for the same site on the apoenzyme[17].

One of the most convenient methods of obtaining selective modification of the C-4-CH_2OH group of pyridoxine is via the use of 3,5'-*O*-dibenzylpyridoxine **127**.



127

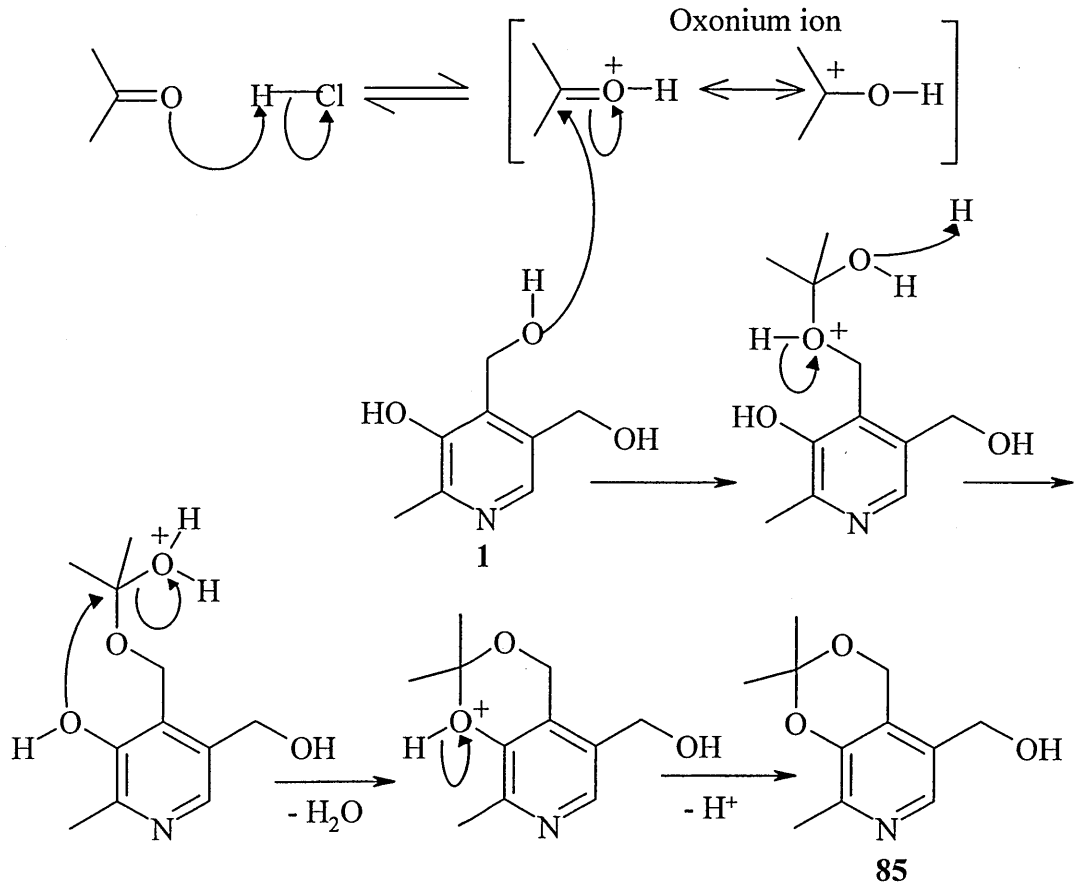
Preparation of this compound begins with the addition of the isopropylidene protecting group at the C-3- and C-4'- hydroxyl groups of pyridoxine. Thus, pyridoxine HCl **1** reacts with acetone saturated with hydrogen chloride gas[132] and the resulting hydrogen chloride salt of 3,4'-*O*-isopropylidenepyridoxine **85** was converted to the free base form by washing with excess saturated $NaHCO_3$ solution. Pyridine **85** was obtained in 92 % yield (scheme 118) as a white solid with mp 99-103 °C, which was slightly lower than the literature mp 110-111 ° C[1]. However, the IR analysis of the compound showed that the peak at 1054 cm^{-1} indicates the presence of the C-O bond. Additionally, the ^{1}HNMR spectrum in $CDCl_3$ revealed that the peaks for the two isopropylidene methyl groups appear as a 6 H singlet at δ 1.55.



1 **85**

Scheme 118

The reaction proceeds in an acid-catalysed nucleophilic addition mechanism and the acidic conditions favoured the formation of the cyclic ketal involving the phenolic group. The acid donates its proton to the acetone to form an oxonium ion, which is highly reactive toward nucleophilic attack at the carbonyl carbon atom as shown in scheme 119.

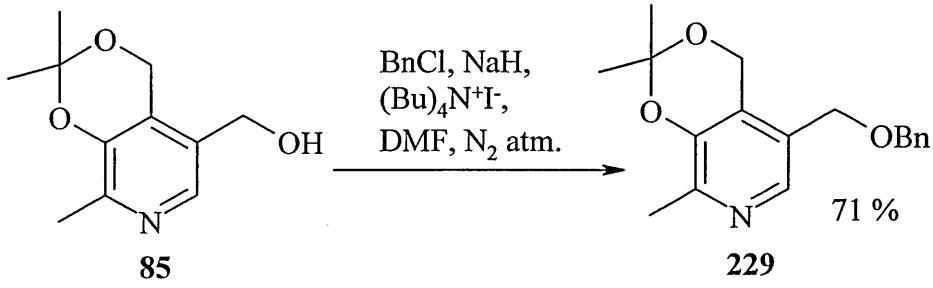


Scheme 119

The 3,4'-*O*-isopropylidenepyridoxine **85** allows the C-5'- hydroxyl moiety to be protected by benzylation. Consequent removal of the isopropylidene group and selective benzylation of the 3-position phenolic group will leave the 4-position CH_2OH group available for modification without affecting neighbouring groups.

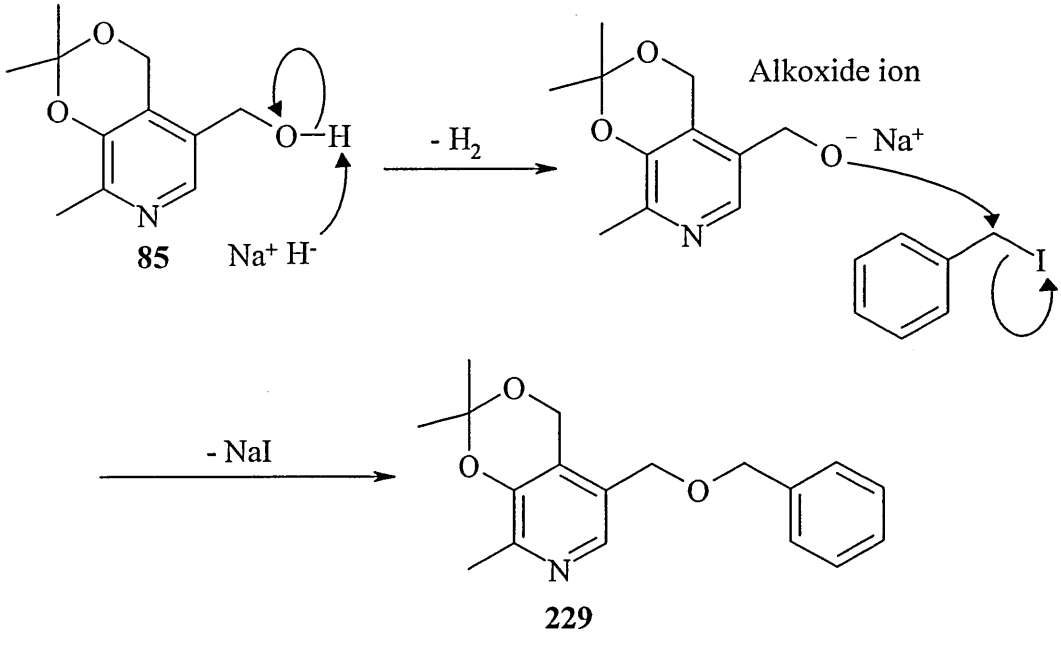
Benzyl ethers as protecting groups of alcohols and phenols are widely used. The ease of removing the benzyl groups by hydrogenolysis, HCl, or trifluoroacetic acid is well known in organic synthesis[214]. Therefore, the C-5'- hydroxyl moiety of the resulting 3,4'-O-isopropylidenepyridoxine **85** was treated with sodium hydride, benzyl chloride and a catalytic amount of *tetra*-n-butylammonium iodide in DMF[1]. The benzylation of compound **85** afforded a yellow oil in 71 % yield after purification by column chromatography. The analysis by IR spectroscopy revealed the apparent peak of the C-O bond at 1143 cm^{-1} and the new aromatic C-H bend peaks at 739 and 699 cm^{-1}. More significantly, the ^{1}HNMR in $CDCl_3$ showed a multiplet at δ 7.22-7.42 indicating the 5 H of the phenyl group and the isopropylidene methyl groups have

moved slightly upfield to δ 1.52. The spectroscopic data were sufficient to confirm that the oil is 3,4'-*O*-isopropylidene-5'-*O*-benzylpyridoxine **229** (scheme 120).



Scheme 120

The benzylation of compound **85** is a classic example of a Williamson synthesis of ether[215]. The reaction is initiated by the C-5'- hydroxyl group of compound **85** reacting with sodium hydride to generate an alkoxide ion. Addition of a catalytic iodide source such as *tetra*-n-butylammonium iodide enables the reaction to overcome the sluggish reactivity of sterically hindered alcohols, toward benzyl chloride, by generating the more reactive benzyl iodide *in situ*. The alkoxide immediately reacts with benzyl iodide by a nucleophilic substitution mechanism (scheme 121).

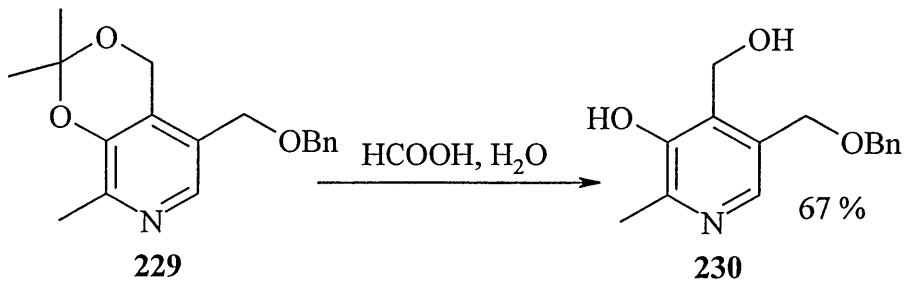


Scheme 121

The mechanism is a typical S_N2 (substitution nucleophilic bimolecular) reaction that takes place in a single step in which the alkoxide ion attacks the carbon bearing the

iodide (leaving group) from the backside and the iodide begins to move away with the pair of electrons. In the transition state, the bond between the oxygen and carbon grows and the bond between the carbon and the iodide weakens which leads to the inversion of configuration of the carbon. The formation of the bond between the oxygen and the carbon atom provides most of the energy necessary to break the bond between the carbon and the iodide resulting in compound **229**.

The 3,4'-*O*-isopropylidene-5'-*O*-benzylpyridoxine **229** undergoes mild acid-catalysed hydrolysis to remove the isopropylidene protecting group. When compound **229** was hydrolysed by heating with formic acid and water[216], a yellow solid was obtained in 67 % yield after purification by column chromatography. The structure of 5'-*O*-benzylpyridoxine **230** was confirmed by the presence of an -OH peak at 3032 cm^{-1} plus the aromatic C-H bend peak at 728 cm^{-1} on the IR. Relevant data from the ^{1}HNMR revealed a 1 H broad singlet at δ 8.40 to be from the phenolic group, a singlet peak for the C-4-C$\underline{H_2}$- group at δ 4.45, and the isopropylidene methyl groups around δ 1.5 no longer present. However, the compound **230** was obtained in 67 % yield only (scheme 122), whereas 94 % yield has been reported in the literature[216]. Lower yield probably occurred during the work up section in the procedure where the pH of the environment can affect the solubility of compound **230** in non-aqueous solvent. Thus, the conditions being slightly acidic can render some of the compound **230** soluble in the aqueous environment.

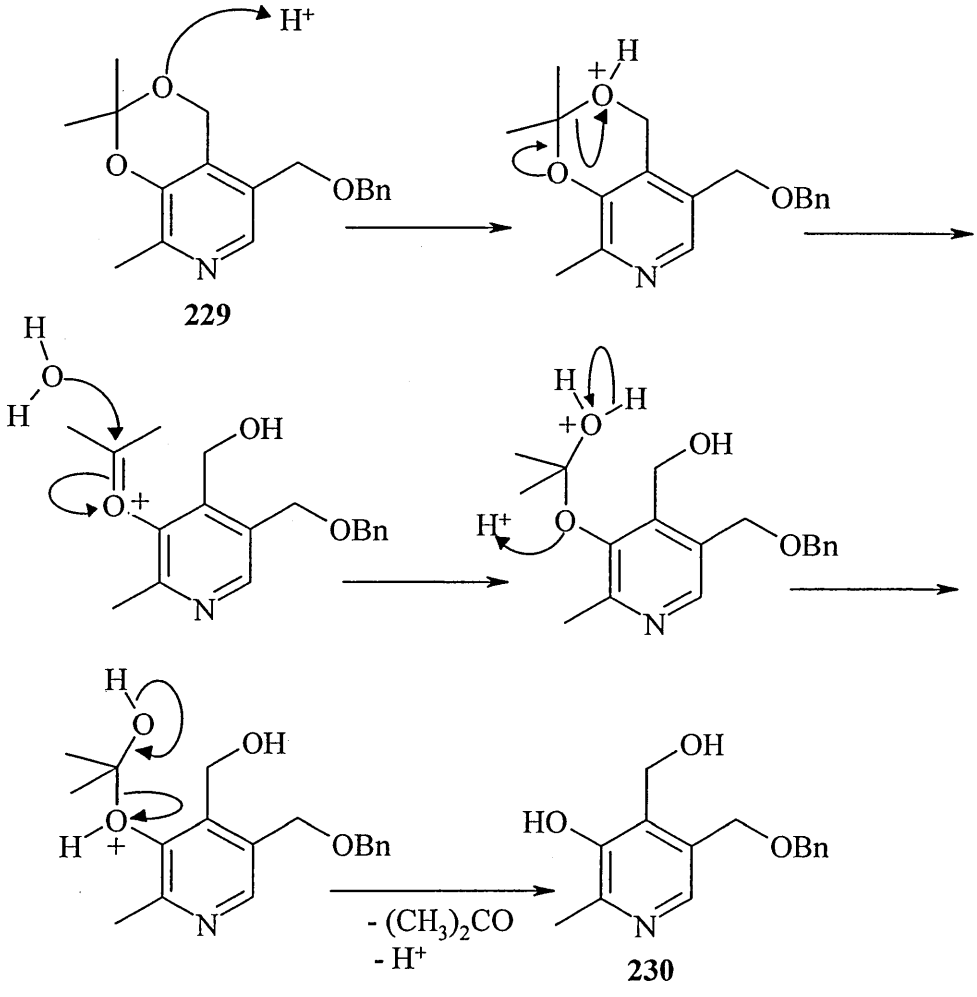


229 **230**

Scheme 122

The hydrolysis of compound **229** was facilitated by the acidic conditions of the reaction. The protonated C-4' oxygen of the cyclic ketal initiates the bond breakage between the carbon and the protonated oxygen. The carbon is stabilised by the transfer of a pair of electrons from the C-3 oxygen to the bond between the carbon and the C-3 oxygen. A molecule of water attacks the temporarily stabilised carbon

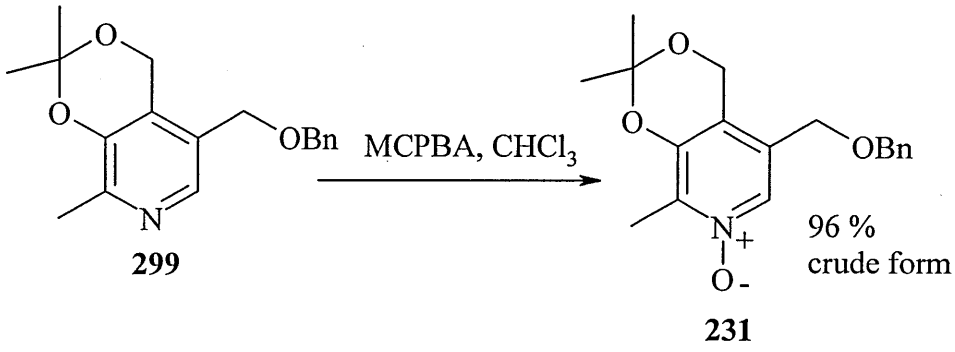
and the pair of electrons returns to the C-3 oxygen. Subsequent protonation of the C-3 oxygen releases acetone and resulted in compound **230** (scheme 123).



Scheme 123

Since the yield of 5'-*O*-benzylpyridoxine **230** was moderately low, another direction was engaged with the investigation of N-oxidation to improve the yield. In addition, it was hoped that N-oxidation would prevent any problems that may arise with the nitrogen of the pyridine in later substitution reactions. 3,4'-*O*-Isopropylidene-5'-*O*-benzylpyridoxine **229** was chosen for N-oxidation as all the functional groups are blocked leaving the nitrogen of the pyridine to be readily oxidised and then hydrolysed afterward to remove the isopropylidene group. When pyridine **229** was N-oxidised with *m*-chloroperbenzioc acid (MCPBA)[217], a brown solid was obtained in 96 % yield as crude. Recrystallisation of a small amount of the crude brown solid from acetone provided a white solid with mp 151-153 °C. The analysis of the white solid established that it is the pure form of the 3,4'-*O*-isopropylidene-5'-*O*-benzylpyridoxine N-oxide **231** (scheme 124) as indicated by the

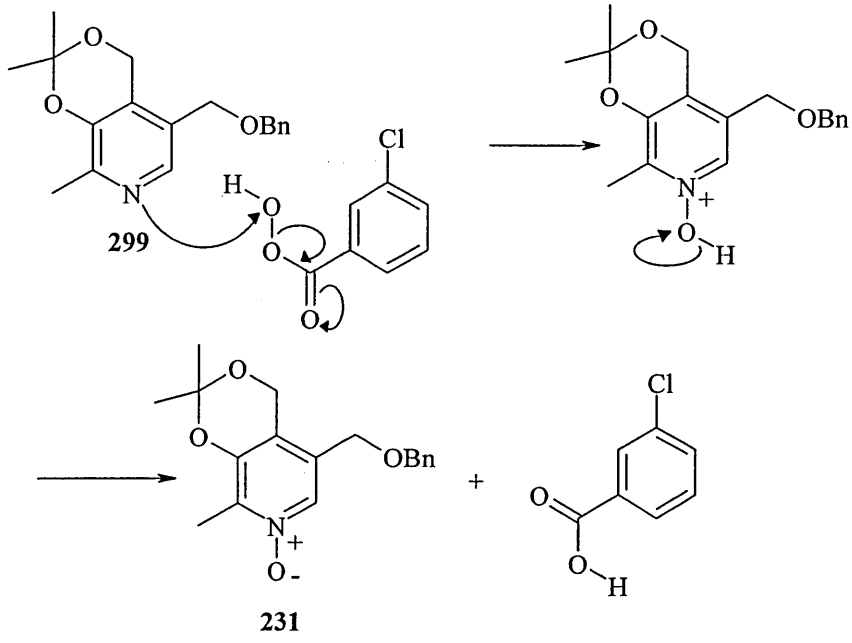
N-O peak at 1209 cm^{-1} and with the aromatic C-H bend peak at 754 and 700 cm^{-1} on the IR. In addition, the ^{1}HNMR revealed that the multiplet peak for the phenyl group is at δ 7.22-7.44. Compared to the non N-oxidised compound **229**, the N-oxide revealed that the singlet peak for the isopropylidene methyl groups had moved downfield to δ 1.58.



299 **231**

Scheme 124

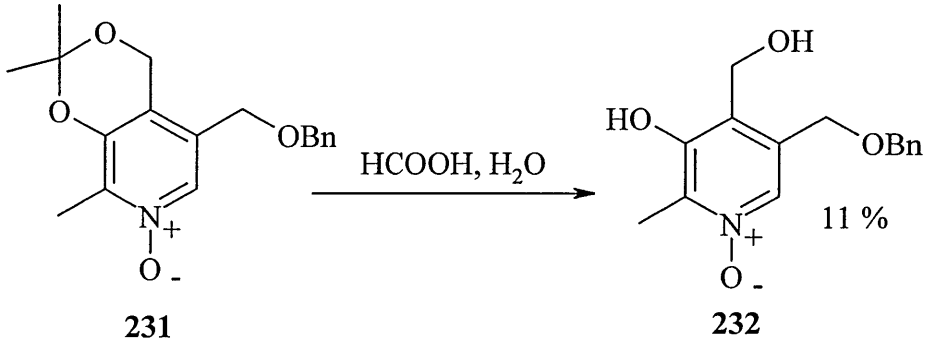
Using MCPBA as the oxidising agent, the nitrogen of the pyridine **299** attacks the MCPBA at the oxygen next to the hydrogen and the *m*-chlorobenzoic acid is lost as a good leaving group to give the N-oxide **231**(scheme 125).



Scheme 125

The 3,4'-*O*-isopropylidene-5'-*O*-benzylpyridoxine N-oxide **231** undergoes mild acid-catalysed hydrolysis to remove the isopropylidene group. However,

reaction of N-oxide **231** with formic acid and water[216] only afforded a white solid in 11 % yield after work up. The IR spectrum of the white solid revealed the presence of a hydroxyl group by the peak at 3030 cm^{-1} and the peak at 1109 cm^{-1} indicates that the N-O bond remained, accompanied by the aromatic C-H bend peak at 741 cm^{-1}. Comparison of the ^{1}HNMR data of the white solid with the non N-oxidised 5'-*O*-benzylpyridoxine **230** revealed downfield shifting of the broad singlet peak for the phenolic group to δ 9.80 and the singlet peak for the C-4-C<u>H</u>$_2$- group to δ 4.49 confirming that the white solid is 5'-*O*-benzylpyridoxine N-oxide **232** (scheme 126). While repeated attempts at the hydrolysis did not gain higher yields of N-oxide **232**, the low yield obtained was probably due to its number of oxygen atoms in the structure, which could lead to its tendency to be more soluble in water and hence the desired product was difficult to isolate. Hence, the poor yield of N-oxide **232** resulted in abandoning any further reactions with N-oxide pyridoxine derivatives.

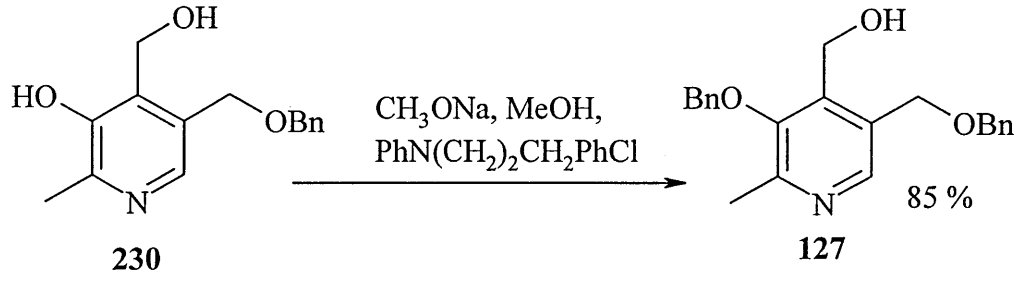


231 **232**

Scheme 126

Given that the N-oxide pyridoxine derivatives were unpromising, attention was focused on the selective benzylation of the C-3 phenolic group of 5'-*O*-benzylpyridoxine **230**. The selective benzylation of the C-3 phenolic group of derivative **230** is achieved by making use of dimethylphenylbenzylammonium chloride reagent **233**. The reagent was prepared as a chloride salt by allowing a mixture of N,N-dimethylaniline and benzyl chloride to stand for several days at room temperature[218]. Therefore, derivative **230** was treated with anhydrous sodium methoxide and reagent **233** in methanol. Purification by column chromatography of the resulting residue provided a yellow solid of 3,5'-*O*-dibenzylpyridoxine **127** in 85 % yield (scheme 127) with mp 62-66 °C and the literature melting point is 64-69 °C[1]. The IR of the yellow solid revealed the essential functional groups present in compound **127**, such as the OH group at 3125 cm^{-1}, the C-O bond at 1100 cm^{-1} along

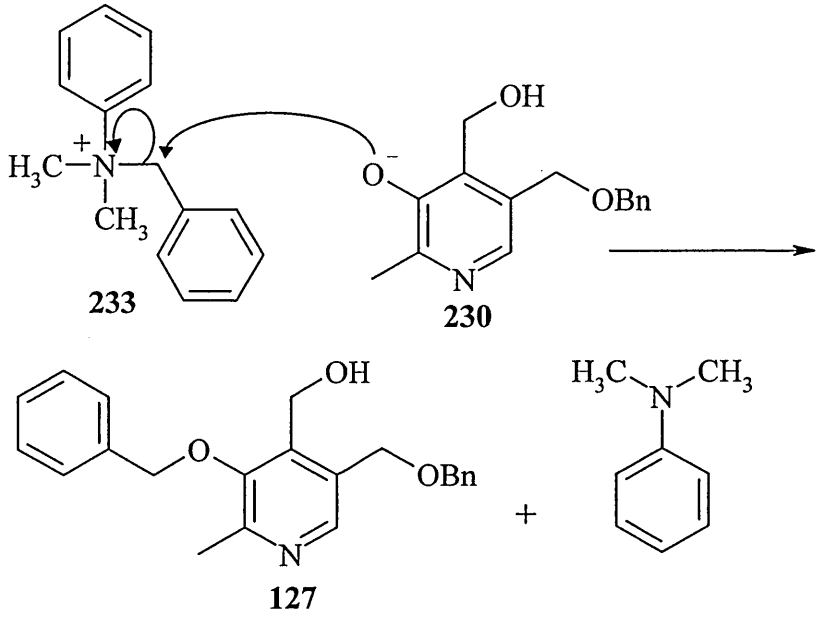
with the aromatic C-H bend at 744 and 696 cm^{-1}. More importantly, the ^{1}HNMR showed the multiplet peak at δ 7.28-7.50 that represents the 10 H of the two phenyl groups and the broad singlet peak at δ 3.34 of the C-4-CH_2O<u>H</u> group.



230 **127**

Scheme 127

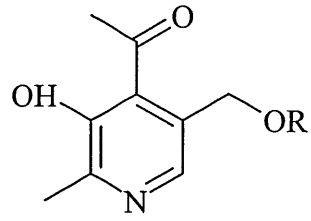
Pyridoxine derivatives benzylated by reagent **233** yield the corresponding 3-*O*-benzyl compound as the only product[219]. In contrast to other alkylating agents, e.g. diazomethane, this reagent does not attack the pyridine nitrogen. The selectivity of the reagent towards the acidic hydroxyl groups (such as phenols and carboxylic acids) can be explained by the formation of an ion pair immediately on the addition of the reagent. On heating, the phenolate anion attacks the electron-deficient benzylic CH_2 group to form the derivative **127** (scheme 128). In order to interact with the dimethylphenylbenzylammonium chloride reagent, 5'-*O*-benzylpyridoxine **230** is first reacted with the base, sodium methoxide, to generate the phenolate anion and the reaction proceeds in a similar fashion as in the Williamson synthesis of an ether (scheme 128).



233 **230** **127**

Scheme 128

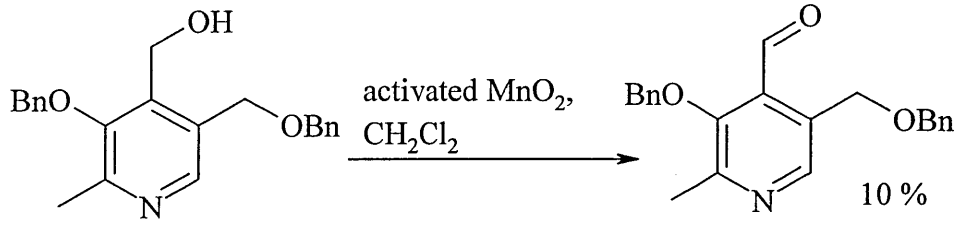
Selective modification at the C-4 position can be achieved by using the 3,5'-*O*-dibenzylpyridoxine **127**, since the benzyl groups have blocked the two neighbouring hydroxyl groups from interfering with later reactions. In this study, the intention was to use pyridine **127** and substitute the C-4-CH_2OH by a C-4-$C(O)CH_3$ group, followed by deblocking of the benzyl groups, to achieve pyridoxine derivatives **234** and with the phosphate group **235**. Oxidation of the C-4' hydroxyl group followed by Grignard reaction and further oxidation would afford the targeted modification.



234 R = H
235 $R = PO_3H_2$

Therefore, 3,5'-*O*-dibenzylpyridoxine **127** undergoes oxidation to convert the C-4' hydroxyl to an aldehyde group and one of the mild reagents for the oxidation of primary and secondary alcohols to carbonyl compounds is manganese IV oxide (MnO_2)[220]. Whether the actual oxidising agent is manganese IV oxide or some other manganese compound adsorbed on the surface of the manganese IV oxide is not clear. However, the advantage of this reagent is that it is specific for allylic and benzylic hydroxyl groups, and reaction takes place under mild conditions (room temperature) in a neutral solvent (water, benzene, petroleum or chloroform). The general technique is simply to stir a solution of the alcohol in the solvent with the manganese IV oxide for some hours. Thus, the pyridine **127** was stirred with activated manganese IV oxide in CH_2Cl_2 at room temperature[217] and the reaction progress observed by tlc analysis at intervals. After stirring for 24 h, tlc revealed a strong presence of starting material even though a new spot was observed that could possibly be the newly oxidised compound. The reaction mixture was stirred for a further 24 h to continue the oxidation process, but the tlc still revealed a spot for the starting material, which was more intense than the spot for the assumed oxidised compound. Thus, the reaction mixture was purified by filtering through a Celite pad followed by column chromatography to afford a light yellow solid in 10 % yield only, whilst unreacted starting material **127** was collected as the major compound in 75 % yield. Analysis of

the light yellow solid by IR revealed the peak of the C-O bond at 1062 cm^{-1} and the aromatic C-H bend peaks at 740 and 698 cm^{-1}. More importantly was the presence of the peak for C=O bond at 1697 cm^{-1} which represents a newly formed aldehyde group. The ^{1}HNMR verified that the light yellow solid is 3,5'-*O*-dibenzylpyridoxal **128** (scheme 129), by the singlet peak at δ 10.37 which represent the hydrogen of the aldehyde group and the multiplet peak at δ 7.12-7.48 for the 10 H of the two phenyl groups.

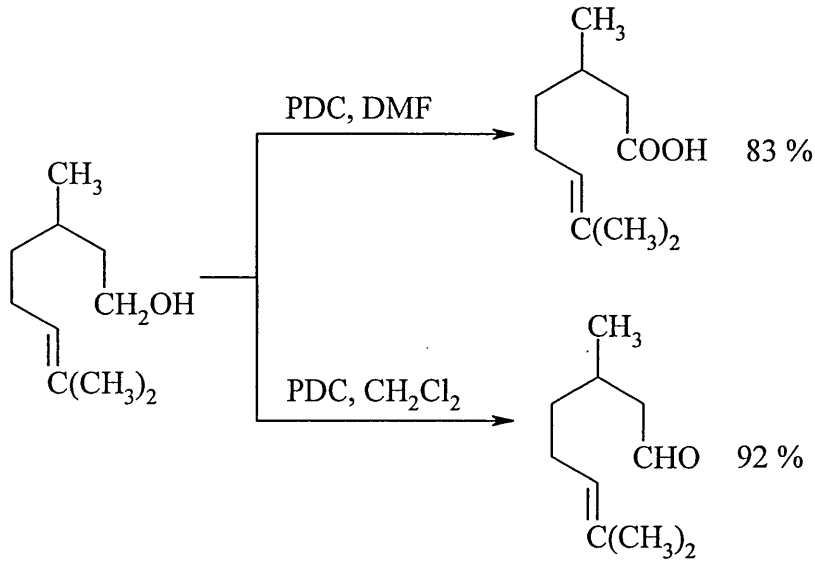


127 **128**

Scheme 129

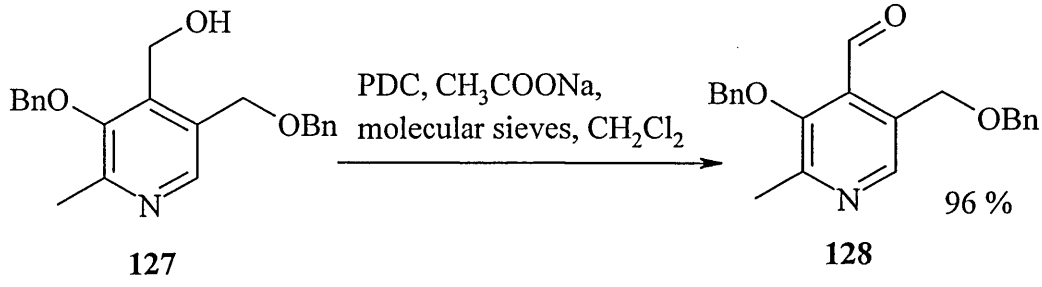
Many factors during the reaction could contribute to the disappointing yield of compound **128** obtained from manganese IV oxide oxidation. Factors such as manganese IV oxide not being activated fully prior to use, the reaction not given satisfactory amount of time to proceed in oxidising the starting material **127**, and the condition of the solvent could affect the overall reaction. In an attempt to improve the poor yield of compound **128**, the manganese IV oxide oxidation was repeated but with no improvement. Thus, oxidation of pyridine **127** with an alternative oxidant was investigated.

Another common oxidant for conversion of alcohols to carbonyl compounds is pyridinium dichromate (PDC). Pyridinium dichromate is a solid obtained by dissolving chromium VI oxide in a minimum of water, adding pyridine, and collecting the precipitate. Unlike the mildly acidic pyridinium chlorochromate, it is nearly neutral. When used in DMF, pyridinium dichromate oxidises aldehydes and primary alcohols to carboxylic acids. However, allylic primary and secondary alcohols are oxidised only to the α,β-unsaturated carbonyl compounds. The behaviour of pyridinium dichromate in CH_2Cl_2 is considerably different. Primary alcohols are oxidised only to aldehydes. Oxidation of secondary alcohols is also satisfactory, while allylic alcohols are oxidised readily[221] (scheme 130).



Scheme 130

Thus, pyridine **127** was treated with pyridinium dichromate in dry CH_2Cl_2 under N_2 atmosphere, with powered 3Å molecular sieves added to enhance the oxidation rate and a catalytic amount of sodium acetate added to reduce the oxidation time[216]. The reaction mixture was stirred at room temperature and reaction progress observed by tlc analysis at intervals. After 24 h, the tlc revealed an intense spot indicating the newly oxidised compound, which is noticeably separated from the faint spot that corresponds to the starting material. Purification of the crude product by column chromatography afforded a light yellow solid in 96 % yield (scheme 131). Analysis of the light yellow solid by IR and ^{1}HNMR revealed that the data are identical to the 3,5'-*O*-dibenzylpyridoxal **128** obtained from the oxidation using manganese IV oxide. Repeats of the oxidation using pyridinium dichromate afforded satisfactory yields of compound **128**.

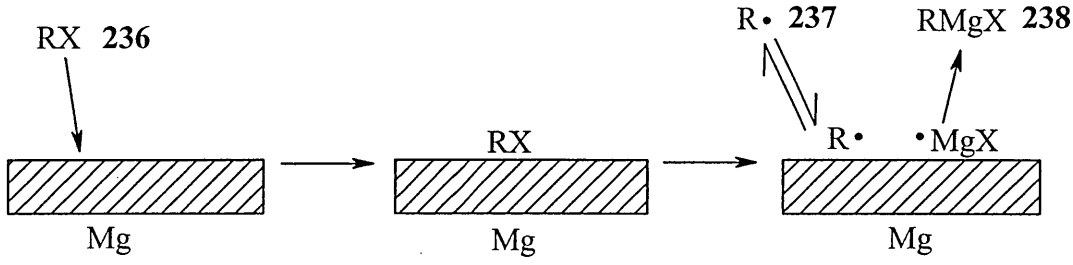


127 **128**

Scheme 131

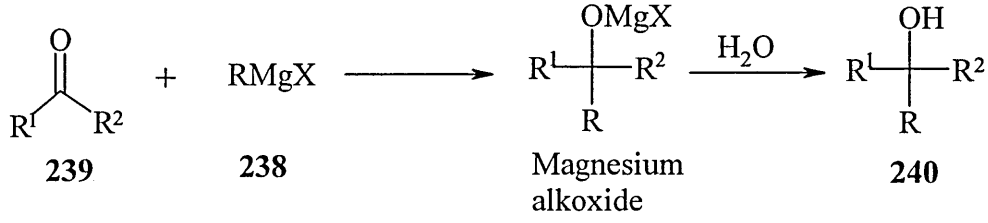
The 3,5'-*O*-dibenzylpyridoxal **128** was now treated with a Grignard reagent to form the secondary alcohol (C-4-CH_2OHCH_3). The reaction of an alkyl or aryl halide

RX **236** with magnesium metal (turnings) in an ether solvent produces organomagnesium compounds of the general structure RMgX **238**. The formation of the organometallic species takes place at the metal surface where the transfer of an electron from magnesium to a halide molecule occurs to form an alkyl or aryl radical species **237**. At the metal surface, the radical species R• and •MgX combine to form the Grignard reagent, which subsequently desorbs from the surface into solution (scheme 132).



Scheme 132

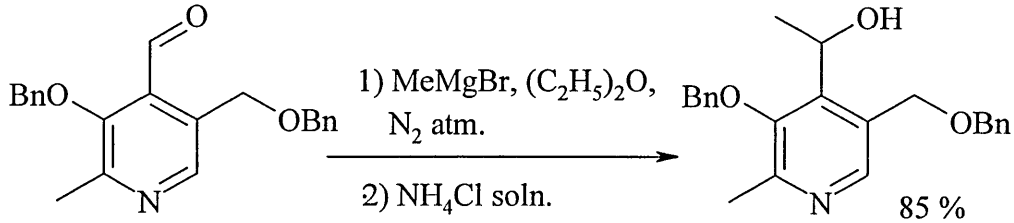
In an alkyl halide, the carbon-halogen bond is polarised, because of the higher electronegativity of the halogen. The carbon centre next to the halogen has a formal δ+ charge, and is electrophilic in its reactivity. In the Grignard reagent however, the carbon centre next to the magnesium is carbanionic in nature and so has a formal δ- charge, thus behaves as a nucleophile. Such a reagent can add to a polarised double or triple bond in the so-called Grignard reaction[222]. Suitable compounds for this versatile reaction are, for example, aldehydes, ketones, esters, nitriles, carbon dioxide, and other substrates containing polar functional groups such as C=N-, C=S, S=O, N=O. Most common, and of major synthetic importance, is the reaction of a carbonyl compound **239**, to give a magnesium alkoxide, which yields an alcohol **240** upon hydrolytic work up (scheme 133).



Scheme 133

Such a reaction occurred readily in the treatment of carbonyl compound **128** with methylmagnesium bromide in ether under N_2 atmosphere[223]. After stirring the

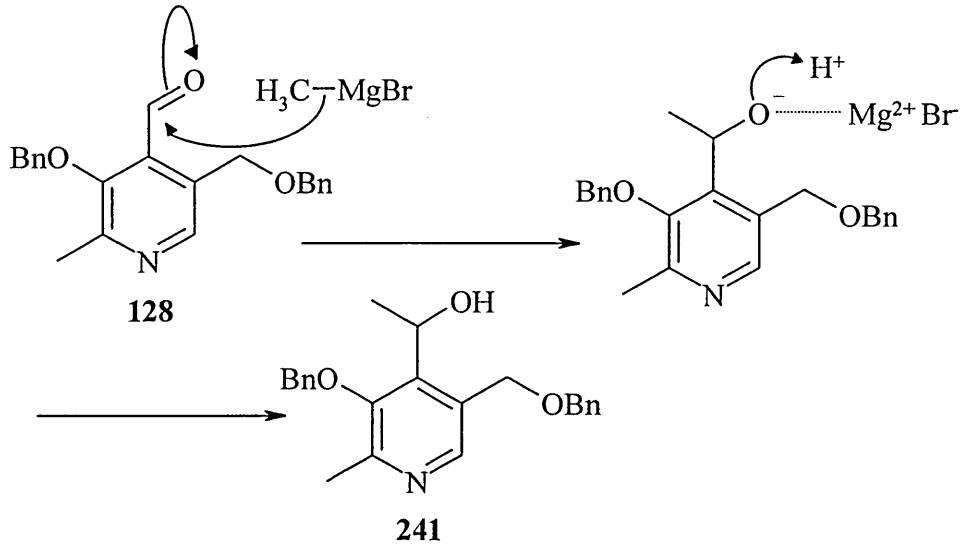
reaction mixture for 4 h, the alkoxide was protonated by addition of dilute aqueous ammonium chloride. IR analysis of the resulting yellow oil (in 85 % yield) after work up revealed the OH group at 3246 cm^{-1}, the C-O bond at 1069 cm^{-1} and the aromatic C-H bend at 737 and 697 cm^{-1}. Further support from the ^{1}HNMR verified that the yellow oil is 4'-methyl-3,5'-*O*-dibenzylpyridoxine **241** (scheme 134), by the presence of a quartet at δ 5.27 (J = 6.7 Hz) as the hydrogen of the secondary alcohol and the 3 H doublet at δ 1.53 (J = 6.7 Hz) of the secondary alcohol methyl group. Also, the obvious multiplet peak at δ 7.28-7.45 indicated the 10 H of the two phenyl groups are present.



128 **241**

Scheme 134

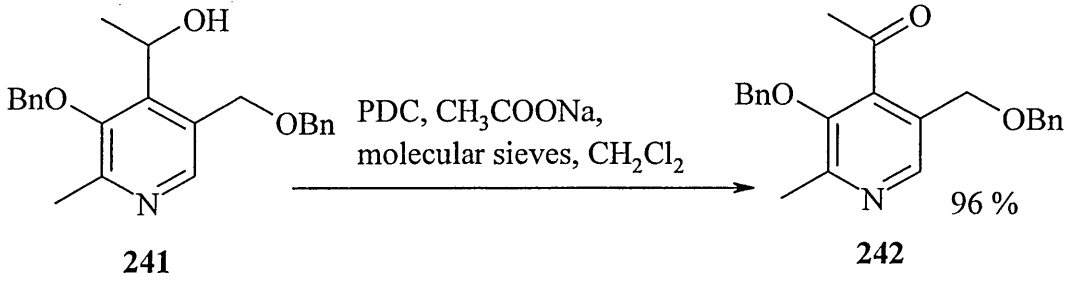
The reaction proceeds where the strongly nucleophilic methylmagnesium bromide uses its electron pair to form a bond to the carbon atom of the carbonyl compound **128**. One electron pair of the carbonyl group shifts out to the oxygen. This nucleophilic addition to the carbonyl group leads to the formation of an alkoxide ion associated with Mg^{2+} and Br^-. The addition of NH_4Cl solution causes protonation of the alkoxide ion and results in the formation of the alcoholic derivative **241** (scheme 135).



128 **241**

Scheme 135

Subsequently, the obtained 4'-methyl-3,5'-*O*-dibenzylpyridoxine **241** was oxidised to convert the secondary alcohol to a ketone group. Compound **241** was oxidised in the same procedure as in the oxidation of 3,5'-*O*-dibenzylpyridoxine **127** by reacting with pyridinium dichromate[216]. The crude residue was purified by column chromatography and the yellow oil obtained in 96 % yield was analysed by IR to reveal the C=O bond at 1706 cm^{-1}, the C-O bond at 1091 cm^{-1}, along with the aromatic peaks at 738 and 699 cm^{-1}. The ^{1}HNMR analysis provided additional evidence which verified the yellow oil is 4'-methyl-3,5'-*O*-dibenzylpyridoxal **242** (scheme 136), such as the 10 H of the two phenyl groups represented by the multiplet peak at δ 7.29-7.40, also, the 3 H singlet of the ketone methyl group at δ 2.53. Particularly noteworthy is the absence of the quartet around δ 5 of the hydrogen of the secondary alcohol.



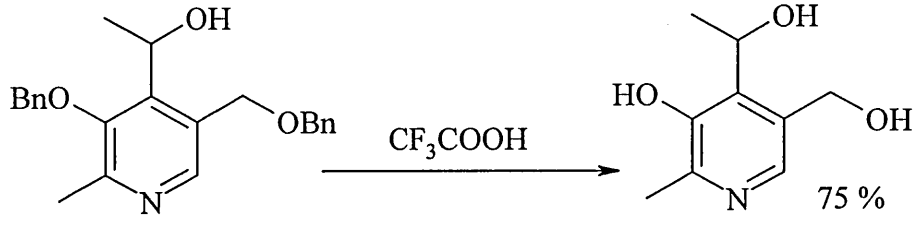
241 **242**

Scheme 136

Removal of the benzyl groups from the protected hydroxyl groups in 4'-methyl-3,5'-*O*-dibenzylpyridoxal **242** was the next objective in achieving the desired derivatives of pyridoxine. Deblocking of the benzyl groups in pyridoxine derivatives could be achieved with the use of HCl, hydrogenolysis with Pd-C, or trifluoroacetic acid. Thus, selecting the appropriate method in removing the benzyl groups from compound **242** was important. Trifluoroacetic acid selectively cleaves benzyl ethers, as well as similar functions like benzylhydryl and trityl, without affecting methyl ethers or ester groups[224]. Thus, compound **242** was treated with trifluoroacetic acid[148] for 24 h, during which tlc analysis was performed at random intervals. Unfortunately, tlc analysis did not show the presence of any compounds apart from a spot for the starting material **242**. As the reaction proceeded, the presence of starting material **242** diminished and a spread of a radiant cloud (under the UV) on the baseline of the tlc plate appeared. This could be due to the polarity of the newly cleaved compound, as pyridoxine itself is soluble in water. Thus, the acid was evaporated from the reaction mixture and the residue purified by ion exchange chromatography eluting with water.

The water fractions were evaporated to a residue and the addition of ether saturated with hydrogen chloride gas afforded a minute amount of an orange precipitate. Intentionally, it was hoped that the precipitation would be a solid once the solvent was removed; however, a sticky residue remained when solvent evaporated. Analysis of the sticky residue by IR spectroscopy remained ambiguous. Repeats of the reaction were unsuccessful. There is the possibility that the cleaved compound had remained on the ion exchange column longer than anticipated and the eluent failed to elute it. Also, it is possible that the presence of water makes it difficult to isolate the cleaved compound since vitamin B_6 derivatives are very soluble in water.

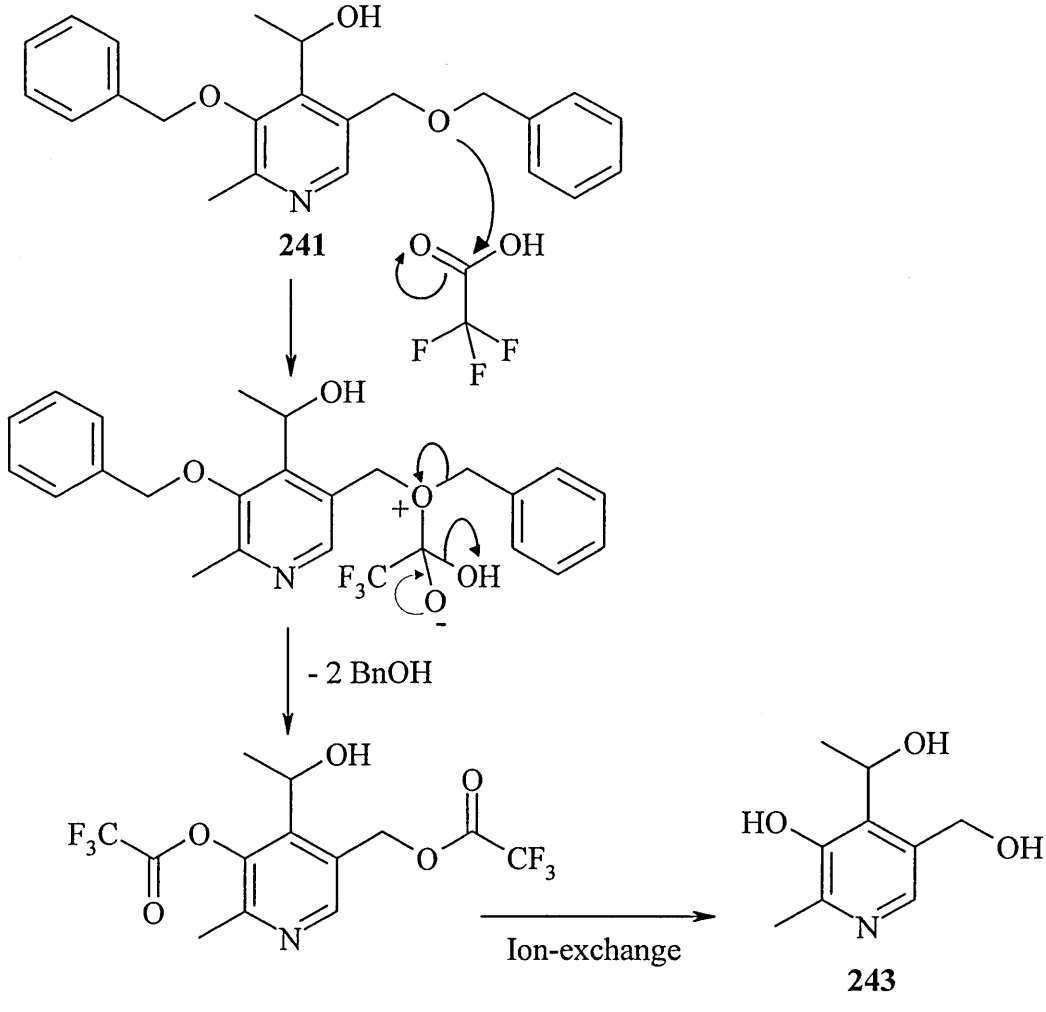
To demonstrate that using trifluoroacetic acid does cleave the benzyl groups from pyridoxine derivatives, 4'-methyl-3,5'-*O*-dibenzylpyridoxine **241** was treated with trifluoroacetic acid[148] and as before the tlc analysis revealed a radiant cloud (under the UV) on the baseline due to the polarity of the newly cleaved compound. The acid was evaporated from the reaction mixture and the residue purified by ion exchange chromatography eluting with water. Similar UV absorption of water fractions were combined and water evaporated. Ether saturated with hydrogen chloride was added to the residue and afforded a cream coloured hydrochloride salt in 75 % yield when the solvent was removed and dried. The cream coloured salt had a mp 176-177 °C while the literature melting point of 4'-methylpyridoxine is in the range of 177-178 °C[223]. Analysis of the cream coloured salt by IR revealed the presence of the hydroxyl groups at 3326 cm^{-1} but the peaks for the aromatics were no longer present. The ^{1}HNMR analysis of the salt in DMSO-d_6 verified that it is the 4'-methylpyridoxine **243** (scheme 137), since a quartet at δ 5.38 (J = 6.7 Hz) due to the hydrogen of the secondary alcohol is present, in addition to the presence of the 3 H doublet at δ 1.43 (J = 6.7 Hz) due to the secondary alcohol methyl group. The obvious 10 H multiplet peak around δ 7, which indicates the two phenyl groups, no longer exists. Repeat of the reaction give a similar yield of compound **243**.



241 **243**

Scheme 137

The reaction proceeds by the oxygen of the benzyl ether attacking the carbonyl carbon of the trifluoroacetic acid. Subsequently, the benzyl group is lost as a cation, which picks up an hydroxyl group. The benzyl group was removed at the 3-position in the same way to produce the trifluoroacetyl derivative. Thereafter, the trifluoroacetyl derivative was subjected to ion-exchange chromatography on a weakly acidic resin, and is hydrolysed to the 4'-methylpyridoxine **243** (scheme 138).



Scheme 138

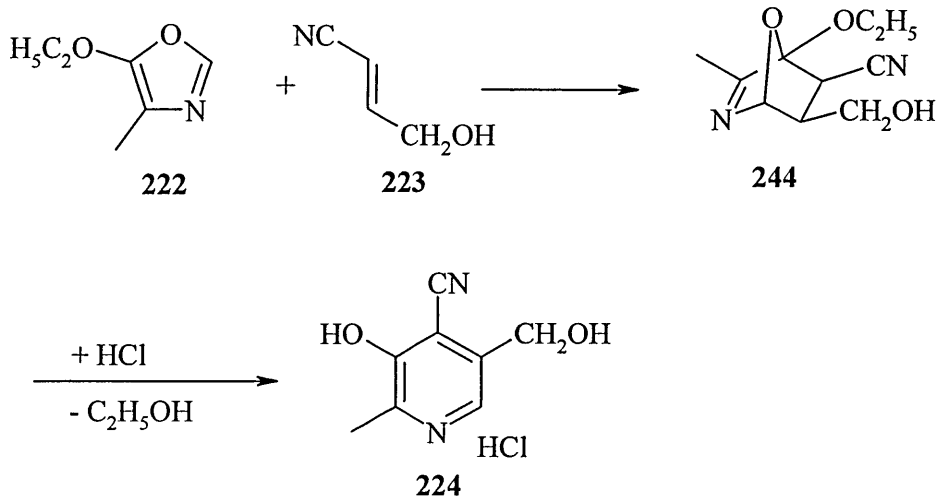
The pyridoxine derivative **243** was treated with a mixture of phosphorus pentoxide and phosphoric acid[24] in an attempt to achieve the phosphorylated form of derivative **243**. After stirring for 4 h in an oil bath at 60 °C, the reaction mixture was purified by ion exchange chromatography, eluting with water. However, IR analysis of the crystals obtained after freeze-drying of the combined fractions remained ambiguous. There is the possibility that the collected crystals are a complex of the

phosphorylating reagent and if the phosphate form was present, the IR spectrum should reveal some resemblance to the starting material **243**.

Attempts at synthesising the phosphate form of 4'-methylpyridoxine **243** were unsuccessful and due to the shortage of starting material further phosphorylation reactions were discontinued. Also, due to the lack of 4'-methyl-3,5'-*O*-dibenzylpyridoxal **242**, additional study on removing the benzyl groups to form the pyridoxal derivative was not possible.

2.2. Total syntheses of vitamin B_6 analogues.

Total syntheses of vitamin B_6 analogues via the Diels-Alder reaction of substituted oxazoles with dienophiles is an alternative route to the modification of commercially available pyridoxine. It was appropriate to select 4-methyl-5-ethoxyoxazole **222** as the azadiene to react with dienophile, 4-hydroxybut-2-enenitrile **223**, to form adduct **244**. Aromatisation of adduct **244** will afford the substituted 3-hydroxy-2-methylpyridine **224** because the $-OC_2H_5$, as a good leaving group, will be eliminated from the adduct (scheme 139).

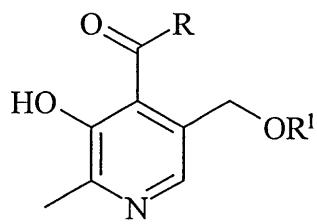


222 **223** **244**

224

Scheme 139

Hence, selective modification can be made at the 4-position of the pyridine **224** by reacting with a Grignard reagent to form the ketone derivative. Subsequent phosphorylation could achieve the phosphate form of vitamin B_6 analogues **220**.



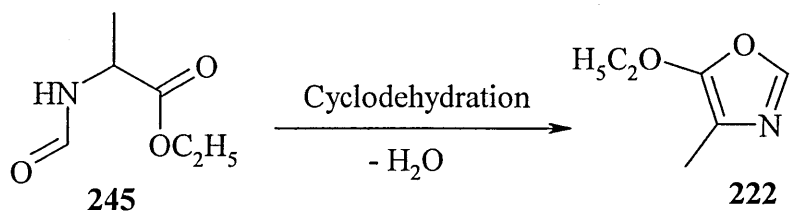
$R = CH_3$, C_2H_5 or longer chain.
$R^1 = H$ or PO_3H_2

220

In order to prepare vitamin B_6 analogues **220**, synthesis of the substituted oxazoles **222** and the dienophiles **223** is required.

2.2.1. Preparation of 4-methyl-5-ethoxyoxazoles.

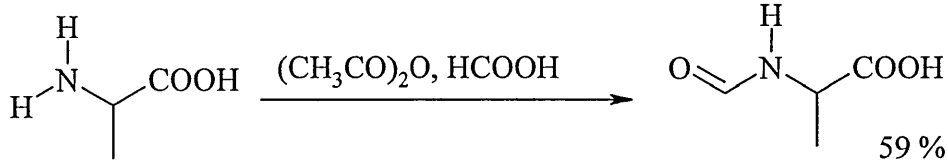
One of the most common routes for preparation of 5-alkoxy oxazoles is via cyclodehydration of α-acylamino carbonyl compounds, known as the Robinson-Gabriel synthesis[158,159], with reagents such as PCl_5, P_2O_5, $SOCl_2$, etc. Hence, N-formyl-(±)-alanine ethyl ester **245** undergoes cyclodehydration to form the 4-methyl-5-ethoxyoxazole **222** (scheme 140).



245 **222**

Scheme 140

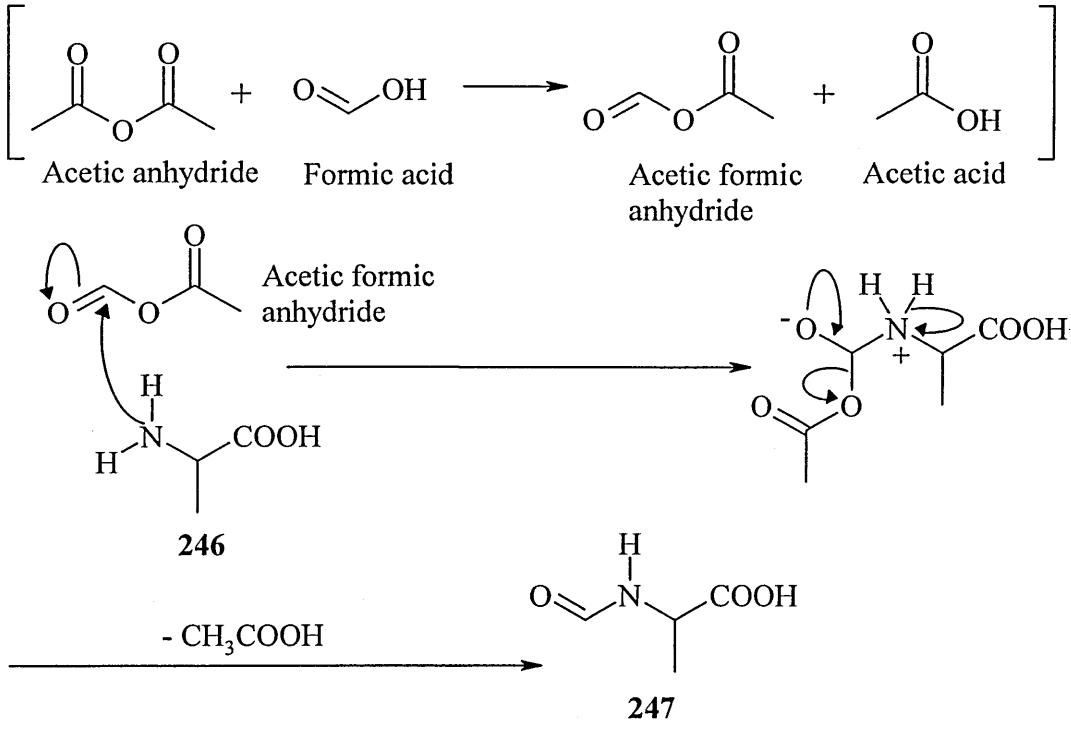
The preparation of the α-acylamino carbonyl **245** began with the N-formylation of (±)-alanine **246** followed by esterification. Reagents such as acetic formic anhydride have been shown to formylate amine derivatives efficiently[225]. Acetic formic anhydrides, usually prepared by gently warming acetic anhydride with formic acid, are used immediately without isolation. Thus, (±)-alanine **246** was treated with acetic formic anhydride[225] to give a white solid in 59 % after cystallisation with ethyl acetate/ethanol (scheme 141). The IR analysis of the white solid revealed the N-H bond at 3355 cm^{-1}, the C=O bond at 1702 cm^{-1}, and the C-H bond peaks at 2854 and 2924 cm^{-1}. The melting point of the white solid in the range of 142-147 °C confirmed that it is the N-formyl-(±)-alanine **247** as the literature melting point is in 145-148 °C[226].



246 **247**

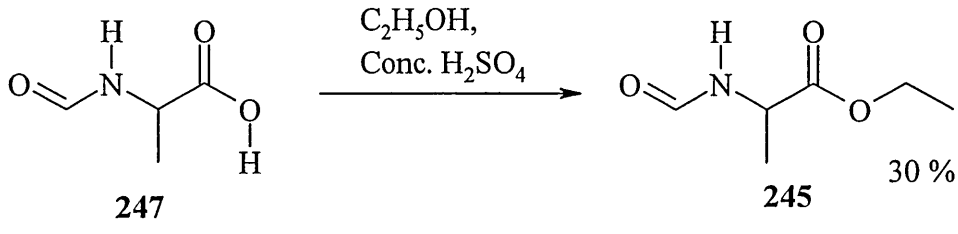
Scheme 141

The N-formylation of (±)-alanine **246** with acetic formic anhydride proceeds via nucleophilic substitution where the nucleophilic amine **246** attacks the formyl carbon of the acetic formic anhydride to form the N-formyl-(±)-alanine **247** as shown in scheme 142.



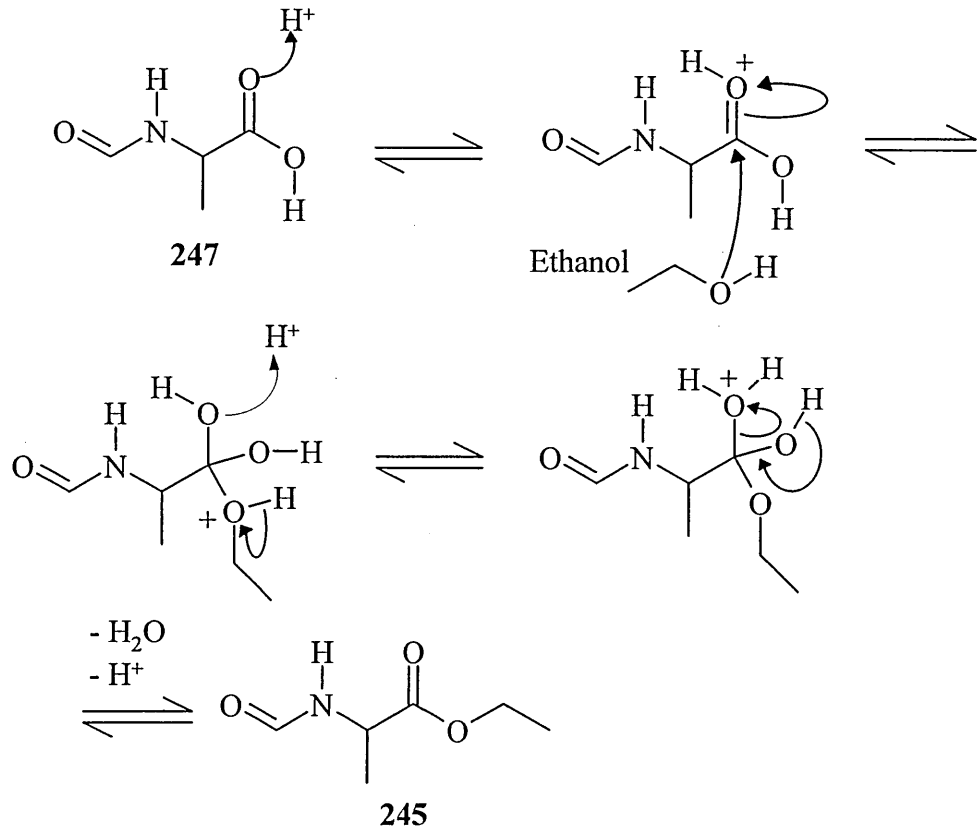
Scheme 142

The N-formyl-(±)-alanine **247** subsequently underwent acid catalysed esterification with ethanol[227] to give the desired N-formyl-(±)-alanine ethyl ester **245** for cyclodehydration. Esterification of N-formyl amino acid **247** using ethanol under acidic condition afforded a residue which was distilled at 110 °C / 3 mm Hg to give a clear oil in 30 % yield only (scheme 143). The literature boiling point of N-formyl-(±)-alanine ethyl ester is 110 °C / 1 mm Hg[226]. The IR analysis of the clear oil indicated the presence of an ester group by the C-O bond at 1205 cm^{-1}, accompanied by the N-H bond at 3300 cm^{-1} and the C=O bond at 1740 cm^{-1} of the N-formyl amino acid ester. The ^{1}HNMR spectrum of the clear oil verified that it is the N-formyl-(±)-alanine ethyl ester **245**. The spectrum revealed the 1 H singlet of the formyl group at δ 8.20, the broad peak of the amido group at δ 7.08 for 1 H, the quartet of the ester -C<u>H</u>$_2$- group at δ 4.22 and the triplet of the ester -C<u>H</u>$_3$ group at δ 1.30.



Scheme 143

In the reaction, the acid catalysed nucleophilic substitution occurs when the carboxylic acid part of **324** accepts a proton from the strong acid and initiates the ethanol attack on the protonated carbonyl group. Consequent loss of a water molecule leads to the ester form of N-formyl-(±)-alanine **245** (scheme 144).

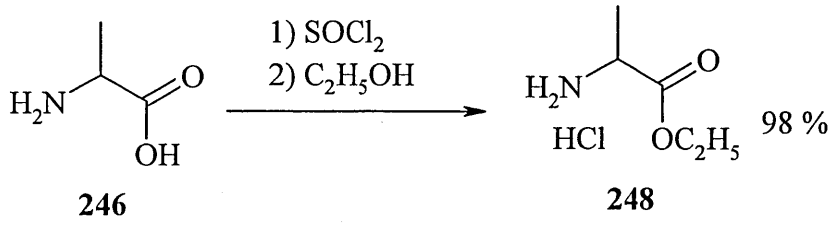


Scheme 144

Since, the esterification reaction is reversible and the presence of water could easily hydrolyse the ester back to the carboxylic acid, hence, the low yield obtained. As the yield of the N-formyl-(±)-alanine ethyl ester **245** is relatively low via this route, a different approach was considered for preparation of the compound **245** in efficient yield for cyclodehydration. Hence, (±)-alanine **246** was esterified first via acyl chloride and then N-formylation afterward.

Therefore, (±)-alanine **246** was treated with thionyl chloride and ethanol[228] and the resulting residue that was precipitated by the addition of ether was collected as a white hydrochloride solid in 98 % yield (scheme 145). The melting point of the white solid is in the range of 78-82 °C which is in close proximity to the literature value of (±)-alanine ethyl ester hydrochloride mp 87-88 °C[229]. Additional information from the IR analysis of the white solid, revealed a peak at 2924 cm^{-1} for the C-H bond and

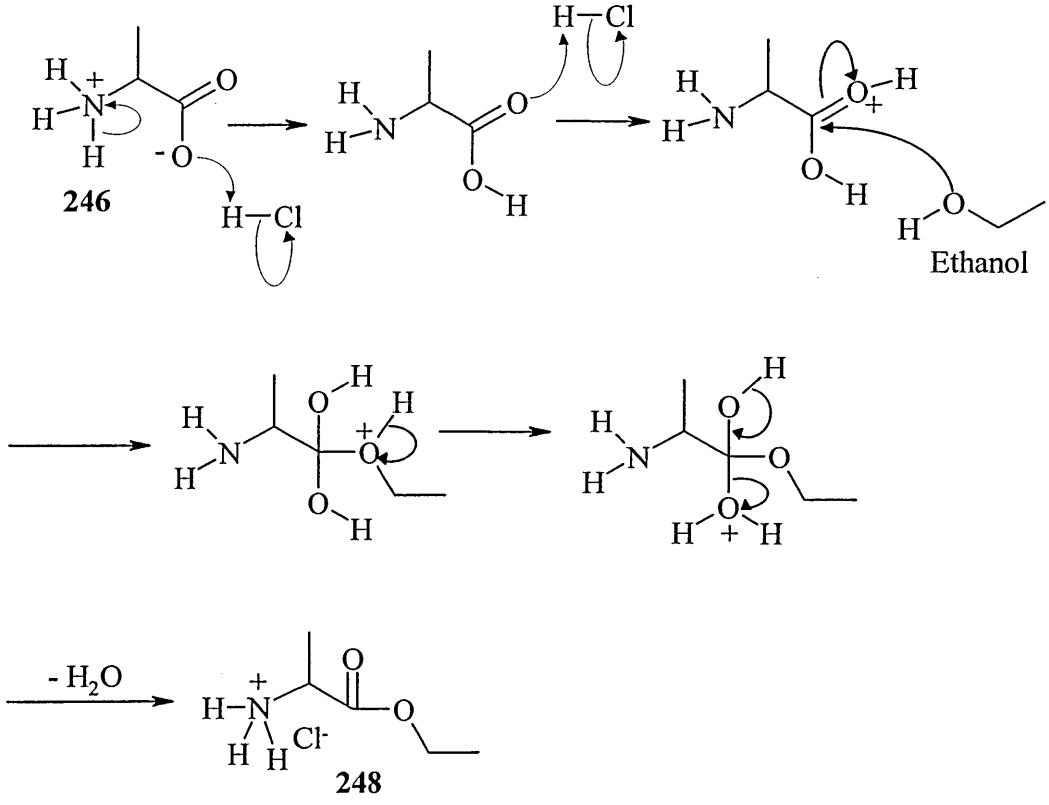
the C=O bond at 1746 cm^{-1} for the ester group, were adequate to verify that it is the (±)-alanine ethyl ester hydrochloride **248**.



246 **248**

Scheme 145

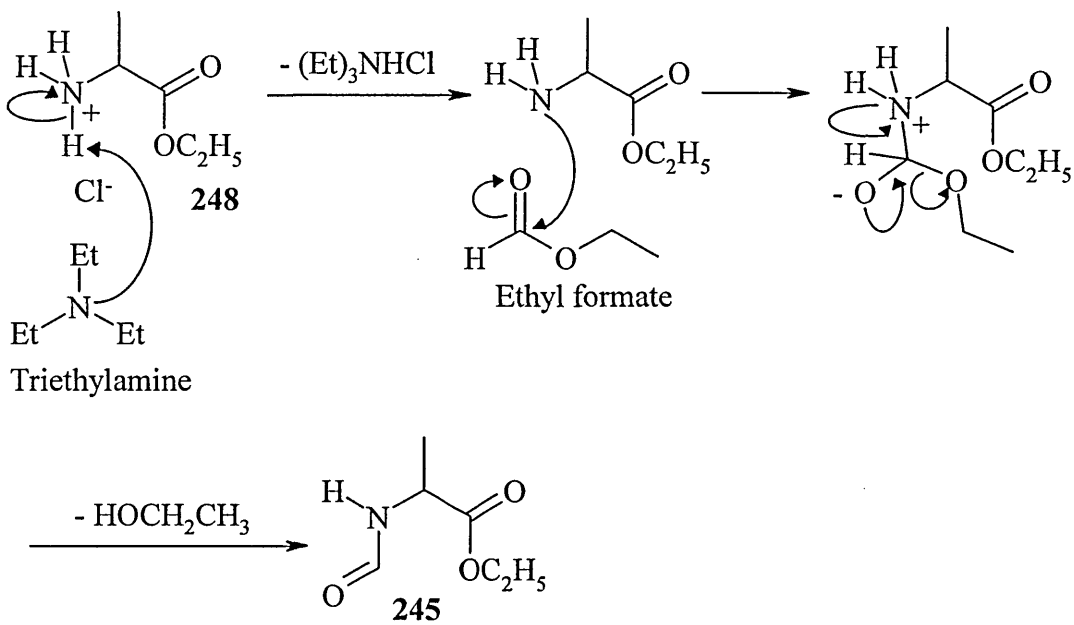
When the reaction was refluxed, the interaction of thionyl chloride and ethanol produces HCl. Accumulation of HCl catalyses the esterification between compound **246** and ethanol to afford the ester compound **248**. The reaction proceeds by the carboxylic acid of compound **246** acceptting a proton from the HCl and then the ethanol attacks the protonated carbonyl group to give a tetrahedral intermediate. Subsequent loss of a molecule of water affords the ester compound **248** as shown on scheme 146.



Scheme 146

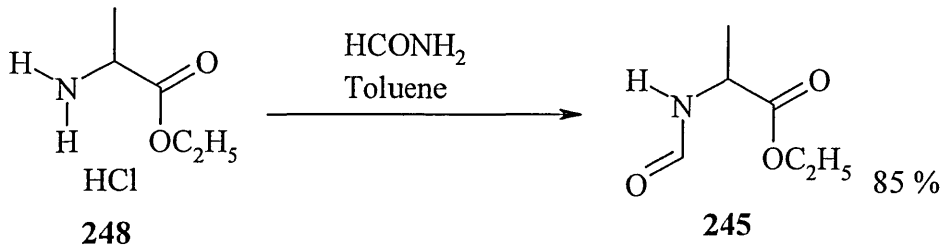
Subsequently, (±)-alanine ethyl ester hydrochloride **248** was subjected to N-formylation to give the N-formyl-(±)-alanine ethyl ester **245**. However, compound **248** in a mixture of acetic anhydride and formic acid, which generates the acetic formic anhydride[225], afforded only a very small amount of clear oil after distilling at 110 °C / 3 mm Hg. The IR spectrum of the clear oil is the same as the spectrum of N-formyl-(±)-alanine ethyl ester **245** from previous synthesis. Inadequate time given for the reaction to proceed or the insufficient generation of acetic formic anhydride could contribute to the extremely low yield.

Another method to N-formylate ester **248**, using triethylamine with ethyl formate and catalytic amount of *p*-toluenesulphonic acid monohydrate[230], also produced a very small amount of clear oil after distilling at 110 °C / 3 mm Hg. Comparing the IR spectra of the clear oil and the α-acylamino carbonyl **245** revealed it to be identical. However, the large accumulation of the triethylamine HCl salt formed in the reaction made the purification process relentlessly difficult and repeated purification was required to remove all the salt fully, consequently, a low yield was obtained. The reaction proceeds by the interaction of the ethyl formate with the primary amine **325** through nucleophilic addition of the amine at the carbonyl of the ester, followed by protonation and elimination of alcohol. The triethylamine serves to free the amine from its hydrochloride (scheme 147).



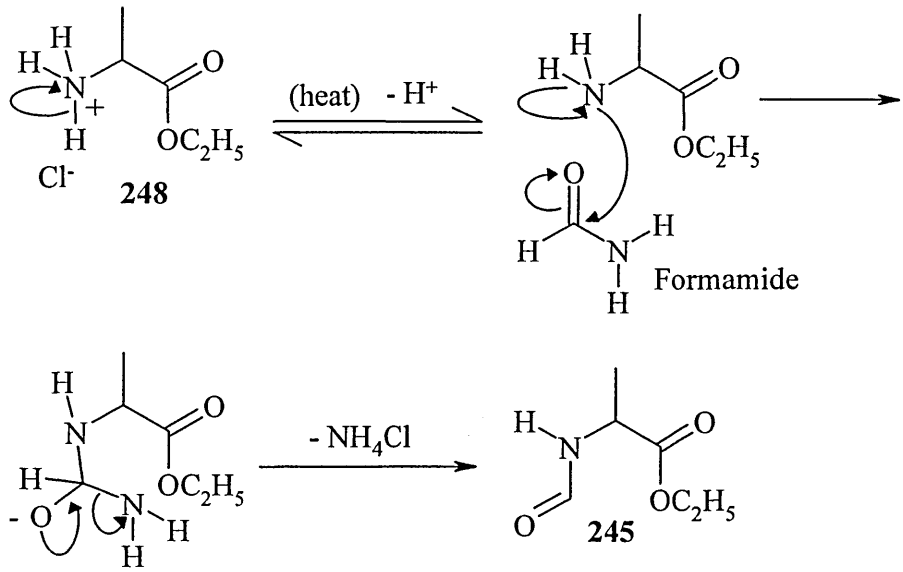
Scheme 147

When ester **248** was subjected to N-formylation using formamide in toluene[231], a clear oil was obtained in 85 % yield after distillating at 110 °C / 3 mm Hg (scheme 148). The IR analysis confirmed that the clear oil is the α-acylamino carbonyl **245**. The reaction proceeds smoothly and fewer problems were associated with purifying the residue obtained, since the NH_4Cl salts formed did not create any difficulties during distillation. Repeats of the reaction produced N-formyl-(±)-alanine ethyl ester **245** in reasonable high yield.



Scheme 148

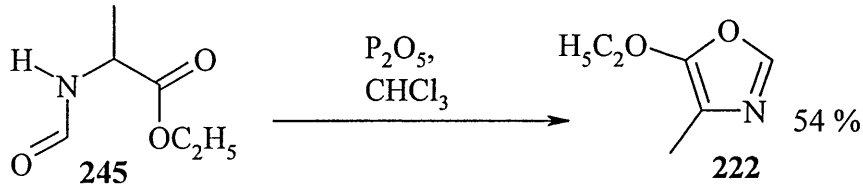
The nucleophilic substitution of the reaction proceeds similarly as in the other N-formylation mechanisms, where the primary amine **248** attacks the carbonyl of the formamide and subsequently eliminates ammonium chloride salt (scheme 149).



Scheme 149

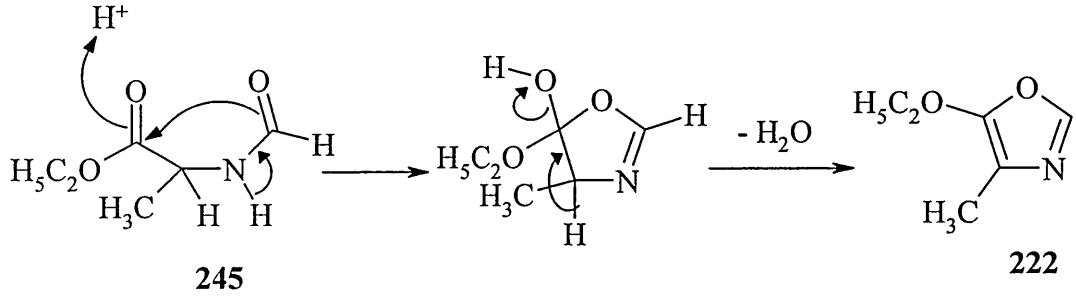
The prepared N-formyl-(±)-alanine ethyl ester **245** undergoes cyclodehydration, known as the Robinson-Gabriel synthesis, to form the 4-methyl-5-ethoxyoxazole **222**. Compound **245** was stirred with cyclodehyrating reagent P_2O_5 in chloroform[232] for 12 h under N_2 atmosphere, and a clear liquid was obtained in 54 %

yield after distilling the residue at 75-80 °C / 50 mm Hg. As the literature boiling point of 4-methyl-5-ethoxyoxazole is at 75-80 °C / 50 mm Hg[232], it seems very likely that the clear liquid is the desired oxazole. The IR analysis of the clear liquid revealed peaks for the C-H stretch at 2929 cm^{-1}, the C-H bend at 1336 cm^{-1}, and the C-O bond at 1221 cm^{-1}. The ^{1}HNMR finally confirmed that the liquid is 4-methyl-5-ethoxyoxazole **222** (scheme 150) by the singlet at δ 7.40 for the -OC<u>H</u>-, the quartet at δ 4.12 for the -C<u>H</u>$_2$O- of the ethoxy group, the singlet at δ 2.04 for the methyl group, and the triplet at δ 1.38 for the C<u>H</u>$_3$- of the ethoxy group.



Scheme 150

The α-acylamino carbonyl **245** cyclised with the elimination of a water molecule to form 4-methyl-5-ethoxyoxazole **222** via the ring closure mechanism of the acylamino double bond attacking the carbon of the ester carbonyl group (scheme 151)[160].

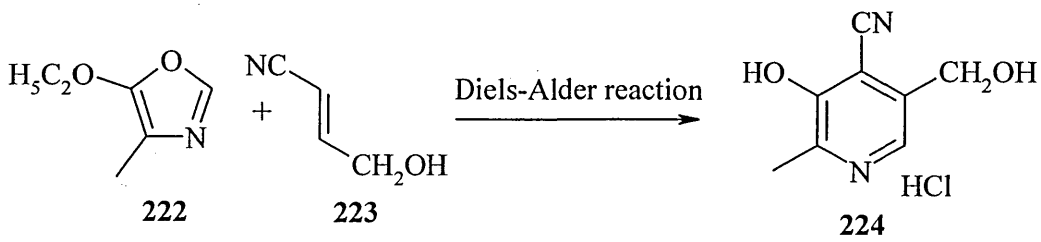


Scheme 151

The 4-methyl-5-ethoxyoxazole **222** was obtained only in modest yield and repeats of the reaction afforded relatively the same amount. An attempt to improve the oxazole yield by subjecting α-acylamino carbonyl **245** to triphenylphosphine with iodine and triethylamine[233] were unsuccessful. As the IR and ^{1}HNMR analysis of the resulting clear oil remained ambiguous and showed no presence of the oxazole **222**. Repeat of the reaction failed to give the desired oxazole, therefore, this approach was abandoned. In this study, the Robinson-Gabriel synthesis using P_2O_5 in chloroform was a convenient method in obtaining the desired oxazole.

2.2.2. *Preparation of 4-hydroxybut-2-enenitriles.*

Since the preparation of the 4-methyl-5-ethoxyoxazoles **222** was satisfactorily completed, the next objective was the synthesis of 4-hydroxybut-2-enenitrile **223** as a dienophile to be utilised in the Diels-Alder reaction with the oxazole **222** to form pyridine **224** (scheme 152).

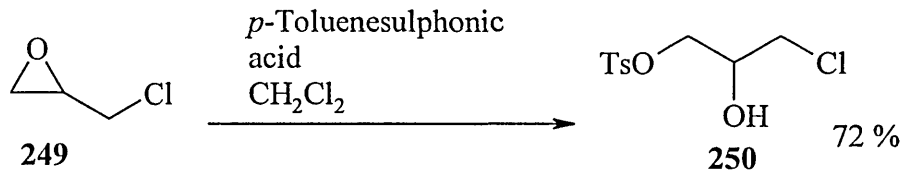


222 **223** **224**

Scheme 152

4-Hydroxybut-2-enenitrile **223** was chosen as the dienophile because in the Diels-Alder reaction the more electron withdrawing substituent (-CN) of the dienophile would end up at the 4-position of the pyridine ring. Hence, by blocking the neighbouring hydroxyl groups of the substituted pyridine selective modification at the 4-position can be conducted.

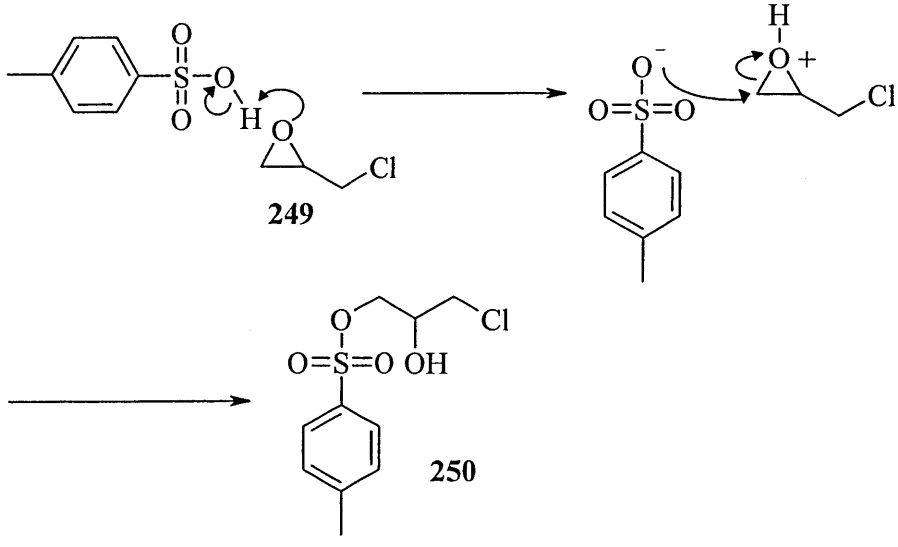
The preparation of the dienophile **223** began with epichlorohydrin **249** which on reaction with *p*-toluenesulphonic acid underwent opening of the epoxy ring[234]. The reaction was refluxed for 3 h to obtain a residue and subsequent purification by column chromatography provided a yellow oil in 72 % yield. The IR analysis of the oil revealed peaks for the hydroxyl group at 3515 cm^{-1}, the alkyl C-H stretch at 2959 cm^{-1}, the C-H bend at 1358 cm^{-1}, and the 1,4-distributed aromatic =C-H bend at 815 cm^{-1}. The ^{1}HMNR spectrum of the oil established it as the desired 1-chloro-3-(toluene-4-sulphonyl)propan-2-ol **250** (scheme 153). The ^{1}HNMR analysis showed the two doublets at δ 7.80 and 7.32 representing the two pairs of aromatic hydrogens, a multiplet in the range of δ 3.50-4.20 corresponding to the underlined hydrogens of Cl$\underline{CH_2}\underline{CH}(OH)\underline{CH_2}$-, a doublet at δ 2.80 for the hydroxyl group, and a singlet at δ 2.45 for the methyl group of the tosyl group.



249 **250**

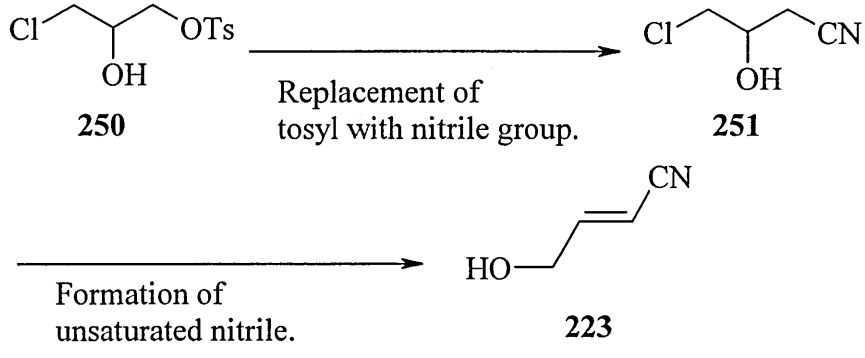
Scheme 153

In the reaction, the epoxide **249** initiates the protonation of the epoxide oxygen that leaves the *p*-toluenesulphonate ion to attack the unsubstituted carbon atom of the epoxide, causing ring opening (scheme 154).



Scheme 154

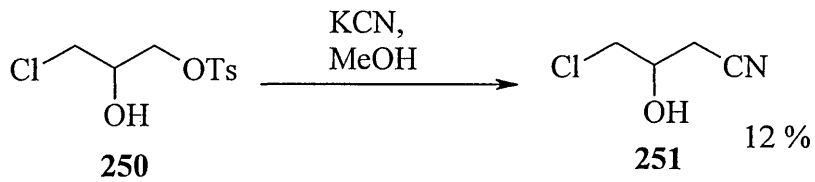
Replacement of the tosyl group of 1-chloro-3-(toluene-4-sulphonyl)propan-2-ol **250** with a nitrile group will give the β-hydroxy nitrile **251**. The β-hydroxy nitrile **251** reacting with a base will eliminate the chlorine and subsequent rearrangement will form the desired α,β-unsaturated nitrile **223** (scheme 155).



Scheme 155

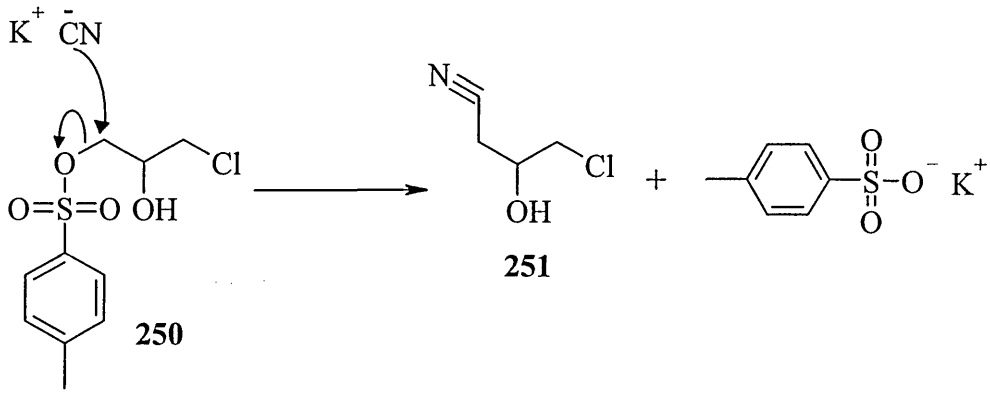
Therefore, the reaction of tosyl derivative **250** with potassium cyanide in methanol[235] afforded a clear yellow liquid in 12 % yield after removal of the potassium tosylate salt and distillation at 115 °C / 4 mm Hg. The IR analysis of the clear yellow liquid showed the presence of a nitrile group by the peak at 2256 cm^{-1} corresponding to a nitrile attached to an alkyl component, accompanied by the hydroxyl group at 3430 cm^{-1} and the C-H stretch at 2962.0 cm^{-1}. Additional

information from the ^{1}HNMR revealed that the liquid is the desired 4-chloro-3-hydroxybutyronitrile **251** (scheme 156). The ^{1}HNMR spectrum shows multiplet peaks at δ 4.28 for -C<u>H</u>-, a doublet at δ 3.65 (C<u>H</u>$_2$Cl), a singlet at δ 3.50 for the -O<u>H</u> group, and a multiplet at δ 2.70 assigned to C<u>H</u>$_2$CN. However, the accumulation of the potassium tosylate salt formed made the purification process difficult and time consuming. Subsequent repetitive filtration and distillation resulted in low yield.



Scheme 156

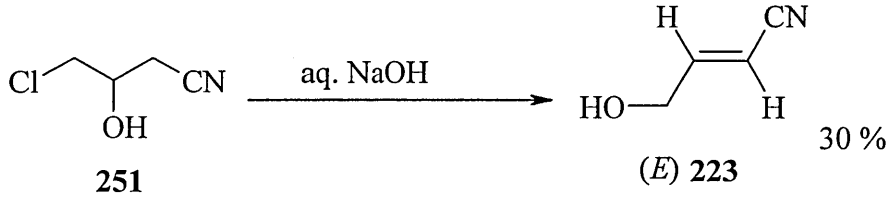
In the reaction, the tosyl derivative **250** underwent nitrilation by reacting with an alkali metal cyanide such as potassium cyanide. Nitrilation proceeds by nucleophilic substitution where the tosyl group is displaced as a good leaving group and replaced by a nitrile group (scheme 157).



Scheme 157

The β-hydroxy nitrile **251** was immediately treated with a base to form the desired dienophile **223**. Thereafter, β-hydroxy nitrile **251** was reacted with aqueous sodium hydroxide[236] solution and the reaction mixture was worked up by continuous extraction. The aqueous solution of the reaction mixture in a round-bottom flask was stirred and heated to boiling. The vaporised aqueous mixture turned to liquid as it reached the condensing tube and flowed to the continuous extraction apparatus where it mixed with ethyl acetate by continuous stirring. Since the ethyl acetate is less dense than water, it, along with the desired compound, gradually floated on top of the stirred solution and flowed back to the original round-bottom flask. Eventually, the desired

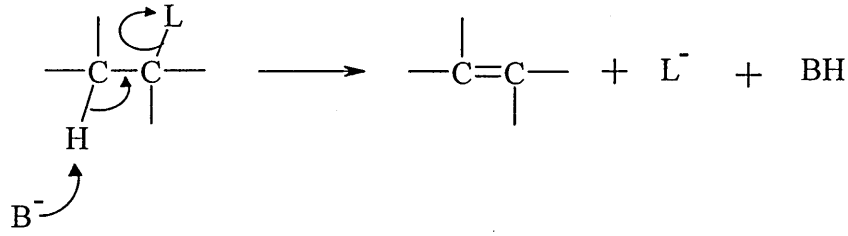
compound in ethyl acetate was collected in the round-bottom flask. The residue, obtained after the removal of the ethyl acetate, was distilled at 136-145 °C / 15 mm Hg to afford a yellow liquid in 30 % yield. The IR spectrum of the yellow liquid revealed the presence of the C=C bond by the peak at 1639 cm^{-1}, along with the hydroxyl group at 3430 cm^{-1}, the C-H stretch at 2920 cm^{-1}, and the nitrile group at 2227 cm^{-1} corresponding to a nitrile attached to vinyl component. The ^{1}HMNR analysis of the liquid showed the doublet at δ 6.92 (J=16 Hz) assigned to the =C<u>H</u>, the doublet at δ 5.68 (J=16 Hz) to =C<u>H</u>, the singlet at δ 4.20 to -C<u>H</u>$_2$-, and the singlet at δ 3.89 to the hydroxyl group. The IR and ^{1}HNMR analysis confirmed that the yellow liquid obtained is (*E*)-4-hydroxybut-2-enenitrile **223** (scheme 158). However, the yield of the product from several attempts was low and in some instances, no product was obtained. The α,β-unsaturated nitrile **223** is soluble in water therefore continuous extraction to collect the compound from the aqueous environment to the solvent phase was necessary and the efficiency of the extraction process would affect the amount of the product obtained.



251 (*E*) **223**

Scheme 158

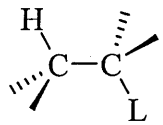
The reaction follows the E2 mechanism of elimination to form the unsaturated compound. The E2 mechanism is a bimolecular 1,2-elimination in which, in a single step, a base removes a proton and a leaving group (L) departs from the compound, resulting in the formation of a π bond (scheme 159).



Scheme 159

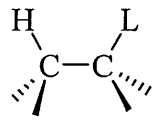
The E2 mechanism is stereospecific[237] where the five atoms involved (including the base) in the transition state must be in one plane. The requirement for coplanarity of

the H-C-C-L unit arises from a need for proper overlap of orbitals in the developing π bond of the alkene that is being formed. There are two ways for this to happen. The H and L may be *trans* to one another (**a**), or may be *cis* (**b**). Conformation (**a**) is anti periplanar and this type of elimination, in which H and L depart in opposite direction, is called anti elimination. Conformation (**b**) is syn periplanar, and this type of elimination, with H and L leaving in the same direction, is called syn elimination.



(**a**)

Anti periplanar

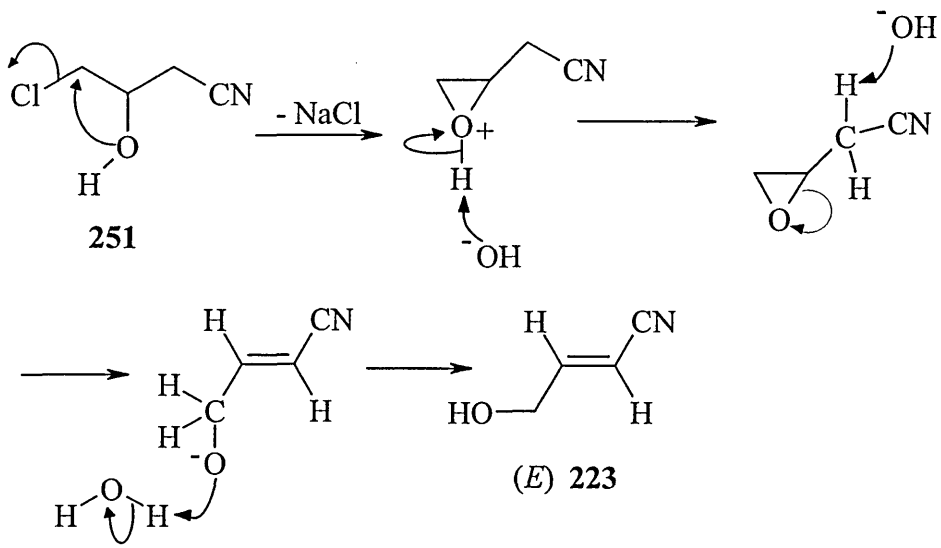


(**b**)

Syn periplanar

The anti elimination is usually favoured over syn elimination because the anti periplanar is a staggered conformation and the molecule requires less energy to reach this transition state than it does to reach the eclipsed transition state of syn periplanar. The syn periplanar transition state occurs only with rigid molecules that are unable to assume the anti rearrangement.

Therefore, the formation of the α,β-unsaturated nitrile **223** under basic conditions allows the β-hydroxy nitrile **251** to form an epoxide by eliminating the chloro group. The formation of α,β-unsaturated nitrile **223** proceeds by an β-elimination mechanism, where the –OH ion attacks a proton from the carbon bonded to the nitrile and simultaneously opens the epoxy ring (scheme 160).

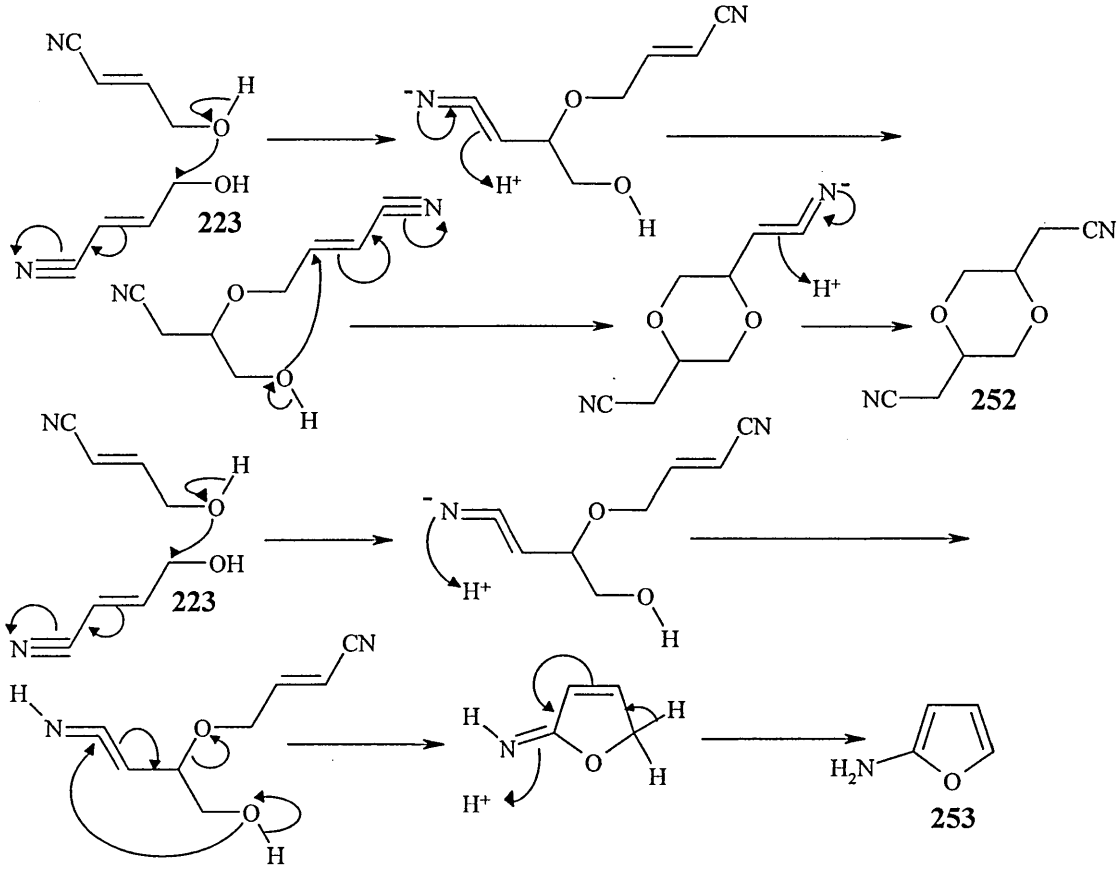


251

(*E*) **223**

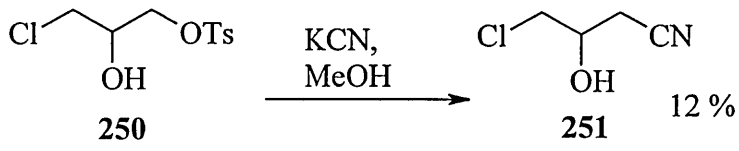
Scheme 160

Crystals which appeared in the yellow liquid of 4-hydroxybut-2-enenitrile **223**, after standing for several days, are due to the reactivity of the compound where it rearranges to form by-products. The 4-hydroxybut-2-enenitrile **223** is known to be remarkably reactive and unstable[236]. Dimerisation occurs easily by the oxygen of the hydroxyl group attacking the β-carbon of another 4-hydroxybut-2-enenitrile molecule to form the 'epicyanohydrin', 2:5-biscyanomethyl 1:4-dioxan **252**. Also, the α,β-unsaturated nitrile **223** has a tendency to cyclise to the extremely unstable 2-aminofuran **253** (scheme 161).



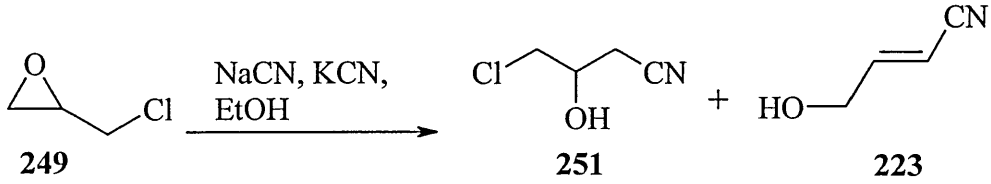
Scheme 161

Since the procedure of using tosyl derivative **250** gave an extremely low yield of 4-chloro-3-hydroxybutyronitrile **251** (scheme 162), it was appropriate to examine other methods in order to obtain a better yield of the compound **251** for conversion into 4-hydroxybut-2-enenitrile **223**.



Scheme 162

An early report used epichlorohydrin **249** directly with a mixture of sodium cyanide and potassium cyanide in neutral conditions[236] to form the β-hydroxy nitrile **251**. An attempt to replicate this method provided a small amount of yellow liquid after continuous extraction with ethyl acetate and distilling at 136-145 °C / 15 mm Hg. The IR analysis of the yellow liquid revealed the presence of β-hydroxy nitrile **251** and of α,β-unsaturated nitrile **223** (scheme 163). During the reaction, the neutrality was maintained laboriously by the addition of standard dilute acetic acid, however, the pH was not always neutral and at a particular period it was basic enough for the formed β-hydroxy nitrile **251** to rearrange to α,β-unsaturated nitrile **223**, as basicity of the reaction favours the formation of α,β-unsaturated nitrile **223**. In addition, if the reaction was slightly acidic the cyanide ions could interact with the acid, giving HCN, and thus a low yield or no product was obtained in repeated attempts.

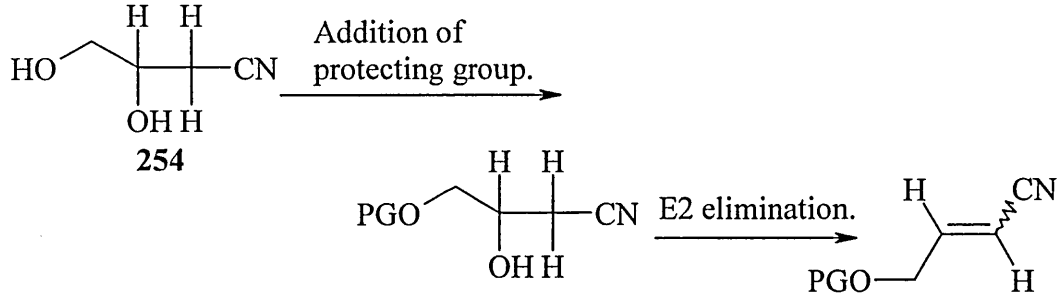


249 **251** **223**

Scheme 163

2.2.2.1. Addition of protecting group.

The 4-hydroxybut-2-enenitrile **223** is extremely reactive and unstable where it dimerises or cyclises to its by-products **252** or **253**. Therefore, it was appropriate to introduce a protecting group into the molecule to reduce the reactivity and instability of the structure. Furthermore, isolation would be a lot easier with a bulky protecting group attached to the molecule. Hence, the addition of a protecting group to the hydroxyl group of β-hydroxy nitrile **254** prior to the α,β-unsaturated nitrile formation might improve the instability of **223** (scheme 164).



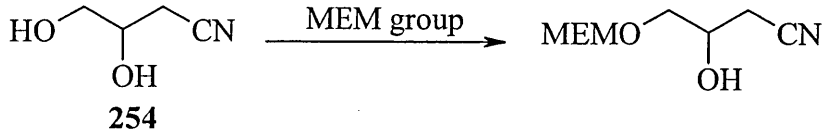
254

PG = Protecting group

Scheme 164

2.2.2.2. The 2-methoxyethoxymethyl ether.

The 2-methoxyethylmethyl (MEM) group is a common protection of the alcohols[214] and would appear to be a suitable functionality for the β-hydroxy nitrile **254** (scheme 165).

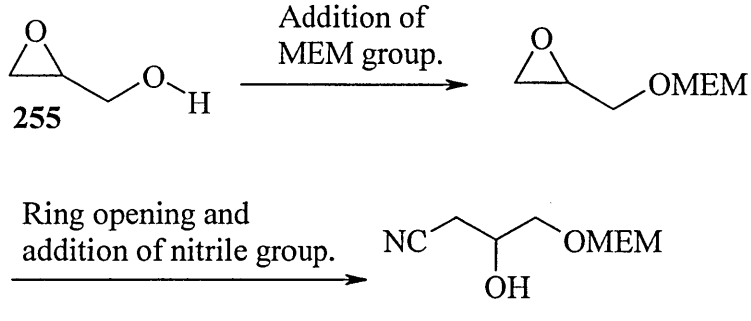


254

Scheme 165

The MEM group can be introduced as 2-methoxyethoxymethyl chloride (MEMCl) to alkoxide ion (prepared from sodium hydride reacting with the alcohol) to afford the MEM ether and can be removed under aprotic conditions. MEM ether is stable under a wide variety of conditions including those attending the use of strong bases, reducing agents, organometallic reagents, many oxidising agents and mild acids.

Since the β-hydroxy nitrile **254** has two hydroxyl groups, the β-hydroxyl group will also form the MEM ether when subjected to the MEM protection. Therefore, glycidol **255** was selected as the appropriate starting material given that the hydroxyl group at the end of the molecule will be MEM protected and subsequent ring opening with the addition of the nitrile group will afford the MEM protected β-hydroxy nitrile (scheme 166).

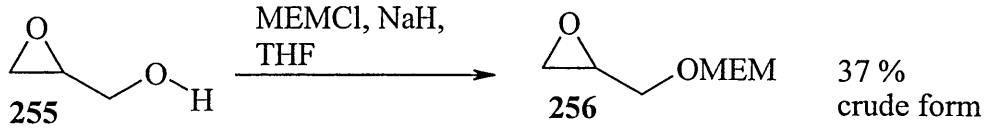


255

Scheme 166

Hence, glycidol **255** was treated with sodium hydride and 2-methoxyethoxymethyl chloride (MEMCl) in THF[238] to give a light yellow oil in 37 % yield after extracting with ethyl acetate and removal of solvent. The IR analysis showed the presence of C-O bond by the peak at 1047 cm^{-1}, also the C-H stretch at 2887 cm^{-1} and the C-H bend at 1458 cm^{-1}. The IR information was sufficient to accept that the 2-(2-

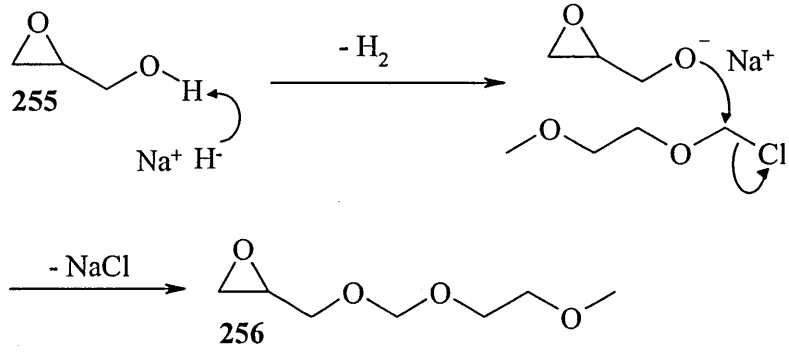
methoxyethoxymethoxy)oxirane **256** (scheme 167) had been obtained and it was used without further purification.



255 256

Scheme 167

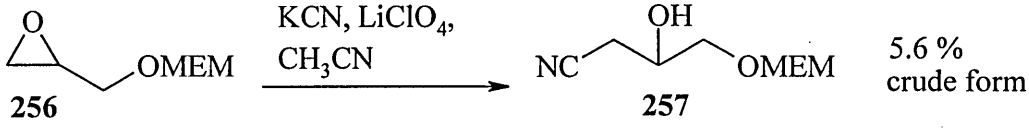
The reaction proceeds with the hydroxyl group of the glycidol reacting with the hydride ion to generate the alkoxide. The alkoxide then reacted with the 2-methoxyethoxymethyl chloride by nucleophilic substitution to form the 2-methoxyethoxymethyl protected glycidol **256** (scheme 168).



255 256

Scheme 168

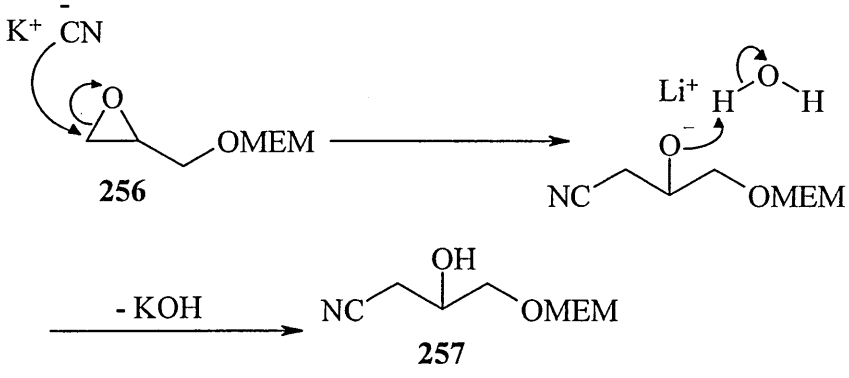
A simple and highly regioselective method for the synthesis of β-hydroxy nitriles is by direct opening of 1,2-epoxides using potassium cyanide in the presence of metal salts as catalyst (such as lithium perchlorate) with non-protic solvent (CH_3CN)[239]. Therefore, the 2-methoxyethoxymethyl protected glycidol **256** was treated immediately with potassium cyanide in the presence of lithium perchlorate in CH_3CN to give a yellow oil in 5.6 % yield after diluting the reaction mixture with water followed by extraction with ether and removal of solvent. The IR spectrum of the yellow oil revealed the hydroxyl group at 3448 cm^{-1}, the C-H stretch at 2934 cm^{-1}, the C-O bond at 1039 cm^{-1}, and most important of all, the nitrile group attached to alkyl component at 2254 cm^{-1}. The IR analysis were sufficient to accept that 3-hydroxy-4-(2-methoxyethoxymethoxy)butyronitrile **257** was formed (scheme 169).



256 257

Scheme 169

Direct formation of β-hydroxy nitriles from epoxides[239] was achieved when the nucleophilic cyanide ion attacked regioselectively on the less substituted epoxide carbon **256**. The metal catalyst coordinated with the epoxide oxygen to form the alkoxide ion. Subsequently, the alkoxide ion then reacted with a hydrogen ion from water to give the β-hydroxy nitrile **257** (scheme 170).



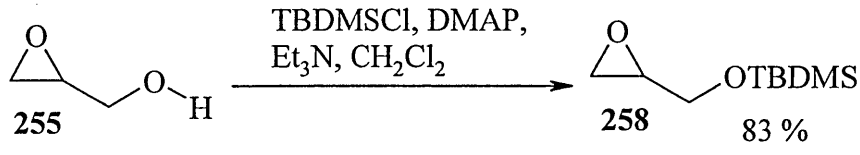
Scheme 170

Due to the extremely low yield of compound **257** it was decided not to do further analytical examination nor to continue further to attempt formation of the α,β-unsaturated nitrile. The reason for the low yield was probably of impurities in the starting material and perhaps loss of the protecting group under the reaction conditions.

2.2.2.3. The tert-butyldimethylsilyl ether.

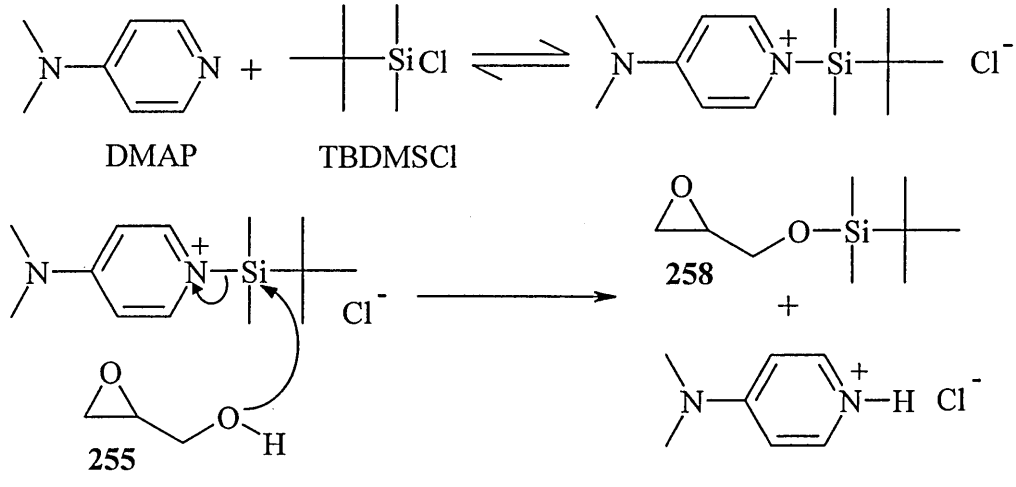
Since the use of MEM group had given an extremely low yields after several attempt. The *tert*-butyldimethylsilyl (TBDMS) group was considered to be a better choice as the group is bulkier than MEM, and therefore might be easier to isolate. The *tert*-butyldimethylsilyl (TBDMS) group are one of the most useful protective groups for alcohols[214]. The TBDMS ether is more stable to hydrolysis than the trimethylsilyl or dimethylisopropylsilyl ether, and cleaved readily by mild acidic hydrolysis conditions. Silylation of the hydroxyl group of glycidol **255** with *tert*-butyldimethylsilyl chloride (TBDMSCl) and imidazole as catalyst in DMF[240] failed to afford the *tert*-butyldimethylsilyl protected glycidol, given that the ^{1}HNMR analysis of the obtained yellow oil showed no sign of the protected glycidol.

However, another method of silylation of glycidol **255** using TBDMSCl , 4-dimethylaminopyridine and triethylamine[241] in CH_2Cl_2 afforded a clear oil in 83 % yield after distilling the residue at 75-80 °C / 2 mm Hg. The IR spectrum of the clear oil showed the presence of alkyl C-H stretch by the peak at 2930 cm^{-1}, the C-H bend at 1472 cm^{-1}, and the C-O bond at 1098 cm^{-1}. Additionally, the ^{1}HNMR analysis verified that the clear oil is 1-*O-tert*-butyldimethylsilyl-2,3-epoxypropane **258** (scheme 171). The ^{1}HNMR spectrum revealed that the two double doublets at δ 3.57 and δ 3.77 are from either one of the hydrogens of the epoxide -C<u>H</u>$_2$O-, along with the multiplet at δ 2.98- 3.04 from the epoxide -C<u>H</u>-. The triplet peak at δ 2.69 and the double doublet at δ 2.55 are from either one of the hydrogens of -C<u>H</u>$_2$-. The singlet peak at δ 0.8 is the 9 H of SiC(C<u>H</u>$_3$)$_3$, and finally the singlet at δ 0.5 represents the 6 H of Si(C<u>H</u>$_3$)$_2$.



Scheme 171

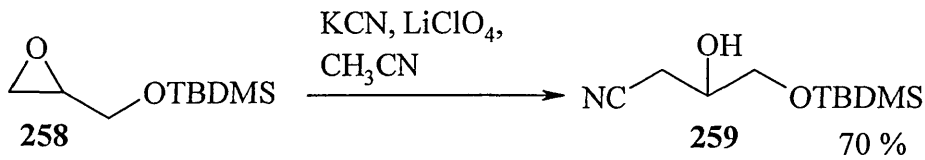
The ability of the 4-dimethylaminopyridine to form a complex with the *tert*-butyldimethylsilyl chloride facilitates the silylation of glycidol, which reacts more efficiently with the hydroxyl group of the glycidol **255** to form the silyl ether **258** as shown on scheme 172.



Scheme 172

Subsequently, the obtained *tert*-butyldimethylsilyl protected glycidol **258** was subjected to direct β-hydroxy nitrile formation using potassium cyanide and lithium

perchlorate in CH_3CN[239] to afford a clear yellow oil in 70 % yield after distilling the residue at 125-130 °C / 3 mm Hg. The analysis of the clear yellow oil verified that it is the desired 4-(*tert*-butylidimethylsilanyloxy)-3-hydroxybutyronitrile **259** (scheme 173) as indicated by the hydroxyl group at 3453 cm^{-1}, the C-O bond at 1122 cm^{-1}, and the nitrile group attached to alkyl component at 2254.6 cm^{-1} on the IR spectrum. In addition, the ^{1}HNMR revealed that the multiplet at δ 3.9 is the C<u>H</u>-, the multiplet at δ 3.7 is the -C<u>H</u>$_2$O-, the doublet at δ 2.72 is the hydroxyl group, and the mutiplet at δ 2.5 is the -C<u>H</u>$_2$CN. The presence of the silyl ether is shown by the singlet at δ 0.85 for the SiC(C<u>H</u>$_3$)$_3$ and by the singlet at δ 0.02 for the Si(C<u>H</u>$_3$)$_2$.

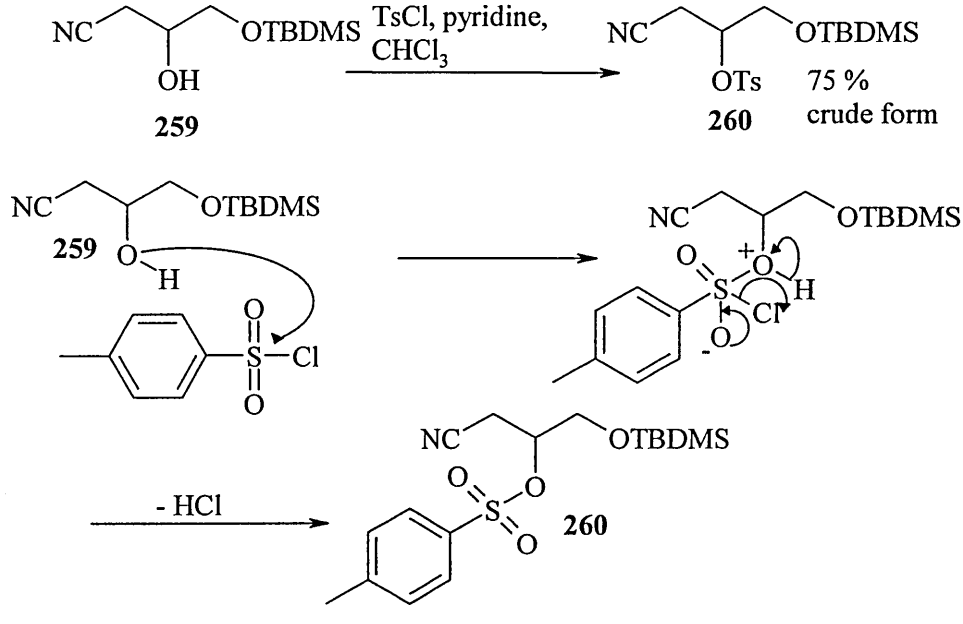


Scheme 173

Repeats of the direct β-hydroxy nitrile formation from derivative **258** afforded the same reasonable yield, therefore, the removal the β-hydroxyl group to achieve the α,β-unsaturated nitrile was considered. The attempts at dehydration of the hydroxyl group of β-hydroxy nitrile **259** to afford the desired α,β-unsaturated nitrile proved to be problematic. Attempted reaction by heating β-hydroxy nitrile **259** with phosphoric acid[242] at 200 °C afforded a dark residue. Further distillation at 130-145 °C / 3 mm Hg of the residue afforded a clear liquid, IR analysis of which revealed the absence of the nitrile group. The high temperature of the reaction probably caused the starting material to decompose. Dehydration of the hydroxyl group of β-hydroxy nitrile **259** with thionyl chloride[243] afforded a small amount of clear liquid after distilling at 70-100 °C / 4 mm Hg, however, the IR spectrum revealed that the nitrile group was also missing. Attempted dehydration of β-hydroxy nitrile **259** with phosphoryl chloride[244] afforded a thick dark brown crude oil after extraction and removal of the solvent. The IR spectrum of the crude showed no sign of the nitrile group. In addition, the reaction of β-hydroxy nitrile **259** with *p*-toluenesulphonic acid monohydrate[245] provided a thick dark brown crude oil after extracting the refluxed reaction mixture and removal of the solvent. The thick crude oil appeared to polymerise on the sodium chloride plates and so an IR spectrum was not obtained. Furthermore, reaction of β-hydroxy nitrile **259** with *p*-toluenesulphonic acid adsorbed on silica gel[246] showed no evidence of the dehydrated compound. The resulting clear oil distilled at 120-150 °C / 4 mm

Hg after column chromatography purification, showed no sign of the nitrile group in the IR spectrum.

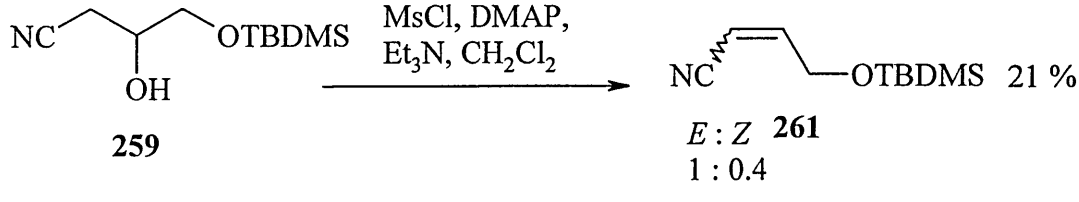
Since several attempts, using different dehydration reagent, to synthesise the desired α,β-unsaturated nitrile were unsuccessful, an approach of using the tosyl derivative to form a better leaving group than hydroxyl group itself was attempted. Therefore, the β-hydroxy nitrile **259**, treated with *p*-toluenesulphonyl chloride[247] and pyridine in $CHCl_3$ at room temperature, provided a white solid crude in 75 % yield after extracting the reaction mixture with ether and removal of the solvent. IR analysis of the white solid revealed the important nitrile group attached to alkyl component at 2254 cm^{-1}, the C-O bond at 1120 cm^{-1}, and the 1,4-distributed aromatic =C-H bend at 839 cm^{-1} on the IR spectrum. Furthermore, the ^{1}HNMR spectrum revealed two doublets at δ 7.30 and 7.91 representing the two pairs of aromatic hydrogens, the multiplet at δ 3.8 is the C<u>H</u>-, the multiplet at δ 3.6 is the -C<u>H</u>$_2$O-, the mutiplet at δ 2.61 is the -C<u>H</u>$_2$CN, and the singlet at δ 2.52 is the methyl group of the tosyl group. The silyl ether protons are shown by the singlet at δ 0.89 for the SiC(C<u>H</u>$_3$)$_3$ and by the singlet at δ 0.02 for the Si(C<u>H</u>$_3$)$_2$. The analytical information was sufficient to confirm that the white solid crude is the toluene-4-sulphonic acid 4-(*tert*-butyldimethylsilanyloxy)-3-(toluene-4-sulphonyl)butyronitrile **260** (scheme 174). The replacement of the hydrogen of the hydroxyl group in β-hydroxy nitrile **259** with the tosyl group involved nucleophilic substitution as shown in scheme 174.



Scheme 174

However, subsequent reaction of compound **260** with potassium hydroxide [248] indicated the presence of starting material after warming the reaction mixture to 40 °C and stirring for 2 days. The tlc analysis of the reaction mixture at random intervals revealed that the starting material still remained and with no indication of a new compound, therefore, the reaction was abandoned. The formation of the α,β-unsaturated nitrile derivative was not achieved due to unsuitable reaction conditions.

Since the α,β-unsaturated nitrile formation via compound **260** were unsuccessful, direct dehydration of β-hydroxy nitrile **259** was attempted using methanesulphonyl chloride[249]. The reaction process is similar to the tosylation of β-hydroxy nitrile **259** except that the intermediate compound was not isolated. The β-hydroxy nitrile **259** was treated with methanesulphonyl chloride and 4-dimethylaminopyridine with triethylamine in CH_2Cl_2 to afford a yellow oil in 21 % yield after distillation. The IR analysis of the yellow oil revealed the presence of the C=C bond by the peak at 1642 cm^{-1}, the C-O bond at 1134 cm^{-1}, and the nitrile group attached to vinyl component at 2225.1 cm^{-1}. The ^{1}HNMR finally confirmed that the yellow oil is 4-(*tert*-butyldimethylsilanyloxy)but-2-enenitrile **261** (E and Z forms in the ratio of approximately 1:0.4) (scheme 175). The E form of compound **261** showed the two double triplets at δ 6.78 (J=16.3 Hz) and at δ 5.68 (J=16.3 Hz) for the two =C<u>H</u>, the double doublet at δ 4.28 for the -C<u>H</u>$_2$O-, the singlet at δ 0.81 for the SiC(C<u>H</u>$_3$)$_3$, and the singlet at δ 0.05 for the Si(C<u>H</u>$_3$)$_2$. The Z form of compound **261** showed the two double triplets at δ 6.41 (J= 11.0 Hz) and at δ 5.30 (J=11.0 Hz) for the two =C<u>H</u>, the double doublet at δ 4.39 for the -C<u>H</u>$_2$O-, the singlet at δ 0.81 for the SiC(C<u>H</u>$_3$)$_3$, and the singlet at δ 0.05 for the Si(C<u>H</u>$_3$)$_2$.



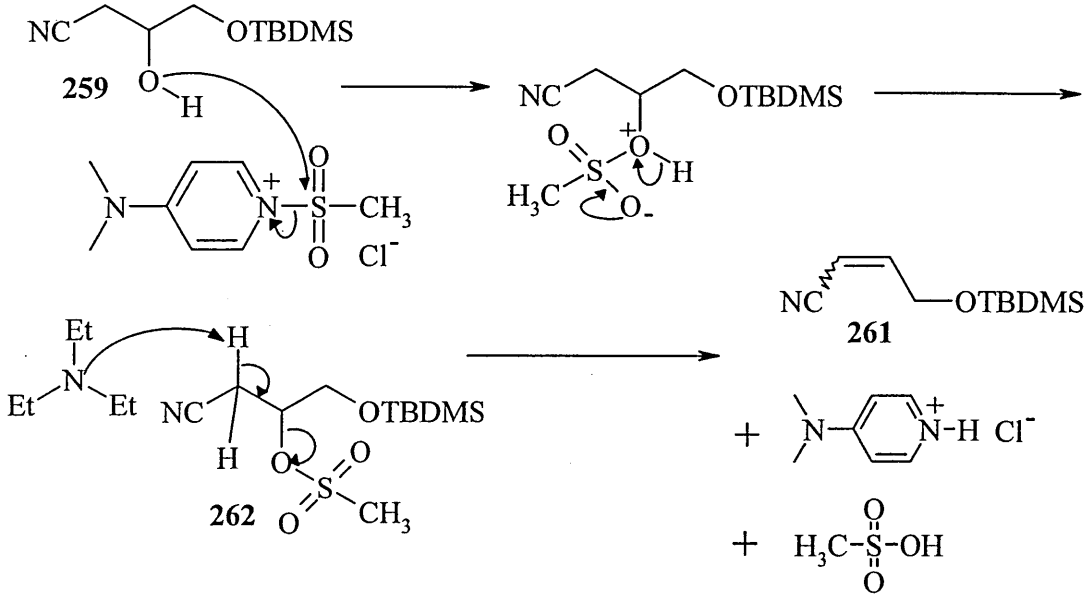
259

E : *Z* **261**
1 : 0.4

Scheme 175

The mesylation of the β-hydroxy nitrile **338** was initiated by the 4-dimethylaminopyridine forming a complex with the methanesulphonyl chloride, which reacts more efficiently with the hydroxyl group. The formed mesyl group was subsequently eliminated from the intermediate **262** when triethylamine attacks the most accessible hydrogen next to the nitrile since the hydrogens close to the TBDMS

ether will be blocked due to steric hindrance, hence, producing the α,β-unsaturated nitrile **340** (scheme 176).



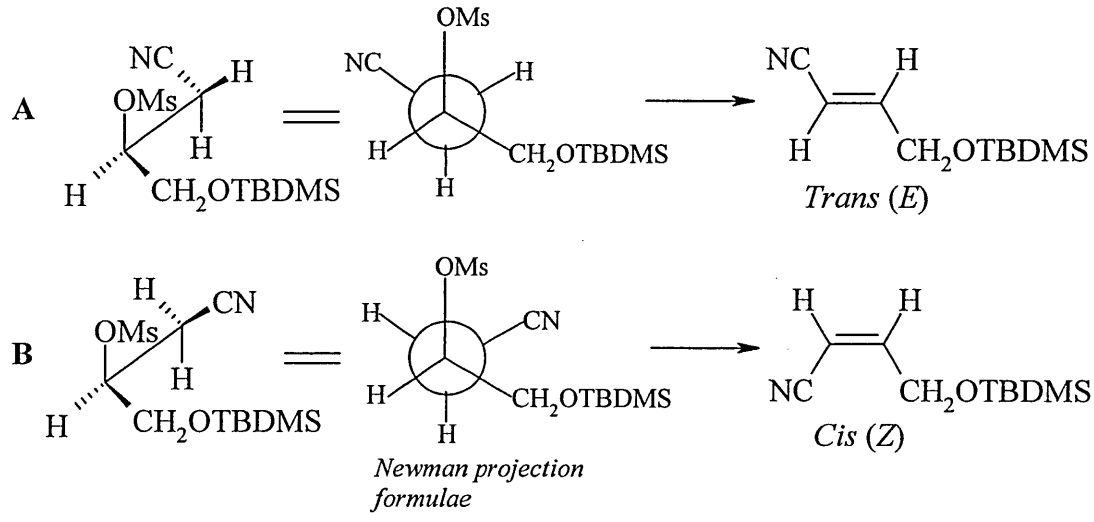
Scheme 176

The elimination process follows the anti E2 mechanism, where the base attacks the proton that is anti periplanar to the leaving group of mesyl derivative **262**, to form the α,β-unsaturated nitrile **261**. When elimination takes place on a compound of the form CH_3–CABX or CHAB–CGGX, the new alkene does not have *cis-trans* isomerism, but for compounds of the form CHEG–CABX (E and G ≠ H) **263** and CH_2E–CABX **264**, *cis* and *trans* isomers are possible[237].

263 **264**

For mesyl derivative **262** two conformations (**A** and **B**) are possible for the transitional state; these lead to different isomers and often both are obtained (scheme 177). In conformation **A** the –CH_2OTBDMS group is between two hydrogens, whereas in conformation **B** the -CH_2OTBDMS group is between the CN and the H. In conformation **B** the electron clouds of the -CH_2OTBDMS group and the CN group are close enough to repel each other, since conformation **A** does not have the eclipsing effect of **B** it is more stable and most of the elimination occurs from this conformation. However, eclipsing effects are not the only factors that affect the *cis/trans* ratio in anti

E2 elimination. Other factors such as the nature of the leaving group, the base, the solvent and the substrate also need to be considered.

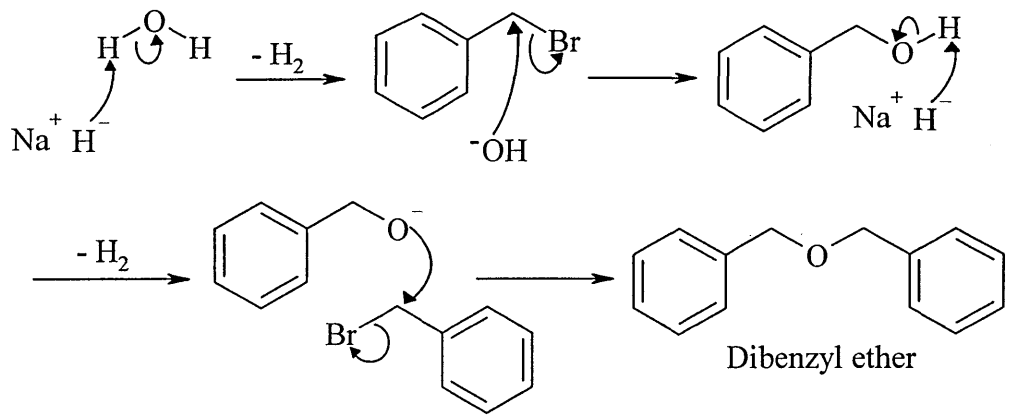


Scheme 177

2.2.2.4. The benzyl ether.

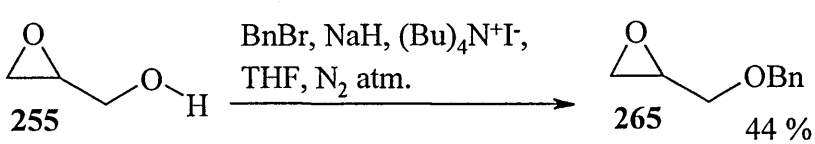
Since the desired protected α,β-unsaturated nitrile **261** was only obtained in low yield (with no improvement after several attempts) it was appropriate to select the benzyl group to protect the hydroxyl of glycidol **255**, since the spectroscopic analysis of the obtained benzyl protected glycidol would be more recognizable due to its aromatic characteristics and yields might be more acceptable. Hence, benzylation of glycidol **255** using benzyl bromide with sodium hydride in THF[216] afforded a clear liquid after distilling at 55-60 °C / 3 mm Hg. The IR analysis showed the C-O bond at 1073 cm^{-1}, also the characteristic peak of the aromatic group at 767 and 698 cm^{-1} for the monosubstituted =C-H bend. However, the ^{1}HNMR spectrum of the clear liquid revealed a 10 H singlet at δ 7.32 of the aromatic group, and a 4 H singlet at δ 4.58 of two -O$\underline{CH_2}$- group. Therefore, the clear liquid obtained is dibenzyl ether and not of the benzyl protected glycidol. The optimum conditions and the presence of water molecules (from the solvent) in the reaction favour the formation of dibenzyl ether. Water molecules in the reaction affect the reactivity of sodium hydride toward glycidol. The sodium hydride reacts with water as well as the alcohol of glycidol. However, the hydroxide ion from the water molecule interacts more favourably with the benzyl bromide than the alkoxide ion of the starting material, hence, the formation of dibenzyl ether dominated the reaction (scheme 178). Another attempt at benzylation of glycidol **255** using benzyl chloride with sodium hydride in DMF[216]

also failed to form the desired benzyl protected glycidol. The reaction afforded a yellow residue after extraction and the IR analysis revealed it to be dibenzyl ether.



Scheme 178

However, the benzylation of glycidol with the addition of *tetra*-n-butylammonium iodide as a catalyst partly overcame the problem of dibenzyl ether formation. The reaction of glycidol **255** with benzyl bromide, sodium hydride and *tetra*-n-butylammonium iodide in THF[250] afforded a light yellow oil in 44 % yield after column chromatography purification. However, dibenzyl ether was also collected in approximately 50 % yield. The analysis of the oil revealed the aromatic C-H stretch at 3031 cm^{-1}, the C-O bond at 1097 cm^{-1}, and the monosubstituted aromatic =C-H bend at 739 and 699 cm^{-1} on the IR spectrum. Furthermore, the ^{1}HNMR analysis revealed a multiplet at δ 7.24-7.37 for the 5 H of the phenyl group, and the two doublets at δ 4.54 for the hydrogens of -OC<u>H$_2$</u>-Ar. The two double doublets at δ 3.42 and at δ 3.77 are due to the two hydrogens of -C<u>H$_2$</u>O-, and a multiplet at δ 3.16-3.22 for the -C<u>H</u>-. Finally, the triplet at δ 2.80 is one of the hydrogen of C<u>H$_2$</u>- and the double doublet at δ 2.61 is due to the other hydrogen of C<u>H$_2$</u>-. The analysis indicates that 2-benzyloxymethyloxirane **265** is the obtained light yellow oil (scheme 179).

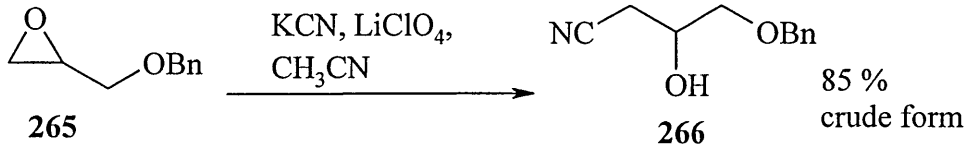


Scheme 179

The formation of benzyl protected glycidol **265** was successful because the catalytic iodide interacts with the benzyl bromide to generate the more reactive benzyl iodide.

The catalytic iodide enables the reaction to overcome the sluggish reactivity of sterically hindered alcohols toward benzyl bromide, by generating the more reactive benzyl iodide *in situ*. Hence, the benzyl iodide is attacked by the alkoxide ion of glycidol and also by the hydroxide ion of water and thus reflects the low yield of the benzyl protected glycidol **265** obtained.

The benzyl protected glycidol **265** was subsequently subjected to direct β-hydroxy nitrile formation using potassium cyanide and lithium perchlorate in CH_3CN[239] to afford a brown oil in 85 % yield after extracting with ethyl acetate and removal of solvent. The spectroscopic analysis of the brown oil confirmed that it was the 4-benzyloxy-3-hydroxybutyronitrile **266** (scheme 180) as indicated by the hydroxyl group at 3444 cm^{-1}, the C-O bond at 1113 cm^{-1}, and the nitrile group attached alkyl component at 2255 cm^{-1}. The aromatic C-H stretch at 3032 cm^{-1}, and the monosubstituted aromatic =C-H bend at 741 and 700 cm^{-1} are also shown in the IR spectrum. In addition, the ^{1}HNMR revealed a multiplet at δ 7.24-7.40 for the phenyl H's, and the two doublets at δ 4.50 for the -OC<u>H</u>$_2$-Ar. The mutiplet at δ 4.05 is the -C<u>H</u>-, and the multiplet at δ 3.50 is the -C<u>H</u>$_2$O-. Finally, the multiplet at δ 2.99 is the hydroxyl group and the multiplet at δ 2.58 is the -C<u>H</u>$_2$CN. Repeats of the reaction afforded β-hydroxyl nitrile **266** in moderate yield.



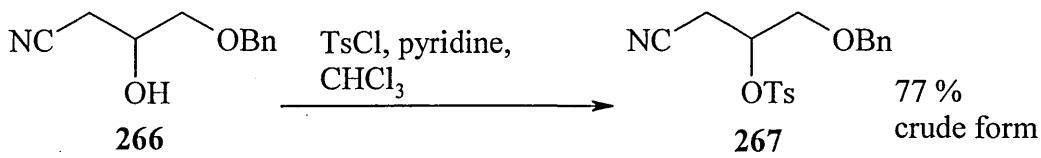
265 **266**

Scheme 180

Attempts at direct dehydration of β-hydroxy nitrile **266** using sodium hydroxide in ethanol failed to provide the desired α,β-unsaturated nitrile compound. After warming the reaction mixture at 40 °C for 6 h, the IR spectrum of the resulting brown oil only showed the presence of starting material. Similarly, the reaction of β-hydroxy nitrile **266** with phosphoric acid[242], refluxed for 30 min., afforded a dark residue after extracting with ether and removal of solvent. The IR spectrum of the residue showed no sign of the nitrile group.

Another approach to α,β-unsaturated nitrile formation is via elimination of the tosyl group. Therefore, the β-hydroxy nitrile **266** was treated with *p*-toluenesulphonyl chloride[247] and pyridine in $CHCl_3$ to give a white semi-solid crude in 77 % yield after extracting the reaction mixture with ether and removal of solvent. The IR analysis of

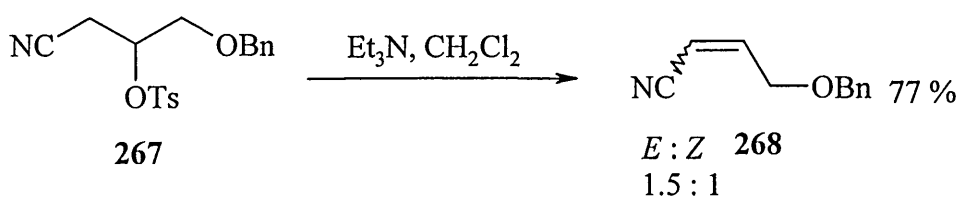
the crude revealed the C-O bond at 1121 cm^{-1}, and the nitrile group attached to alkyl component at 2254 cm^{-1}. Additionally, the aromatic C-H stretch at 3091 and 3061 cm^{-1}, the 1,4-disubstituted aromatic =CH bend at 814 cm^{-1}, and the monosubstituted aromatic =C-H bend at 751 and 702 cm^{-1} are also present. The spectroscopic analysis was sufficient to designate the crude product as the 4-benzyloxy-3-(toluene-4-sulphonyloxy)butyronitrile **267** (scheme 181).



266 **267**

Scheme 181

Compound **267**, when treated with triethylamine[249] in CH_2Cl_2 should undergo α,β-unsaturated nitrile formation since the tosyl group will be eliminated as a good leaving group. The reaction afforded a brown oil in 77 % yield after column chromatography purification. Further purification by distilling a small fraction of the brown oil afforded a yellow oil. Analysis of the yellow oil revealed the aromatic C-H stretch at 3032 cm^{-1}, the C-O bond at 1155 and 1116 cm^{-1}, the monosubstituted aromatic =C-H bend at 738 and 698 cm^{-1}. More importantly, was the presence of a nitrile group attached to a vinyl component at 2223 cm^{-1}, and the C=C bond by the peak at 1670 and 1636 cm^{-1} on the IR spectrum. The ^{1}HNMR confirmed that the yellow oil is 4-benzyloxybut-2-enenitrile **268** (E and Z forms in the ratio of approximately 1.5:1) (scheme 182). The E form of compound **268** showed a multiplet at δ 7.36 for the 5 H of the phenyl group, also the two double triplets at δ 6.74 (J=16.3 and 2.4 Hz) and at δ 5.72 (J=16.3 and 2.4 Hz) for the two =C<u>H</u>. The singlet at δ 4.56 is the -OC<u>H</u>$_2$-Ar-, and the doublet doublet at δ 4.14 (J=1.2 and 2.4 Hz) is the -C<u>H</u>$_2$O-. The Z form of compound **268** showed a multiplet at δ 7.36 for the 5 H of the phenyl group, also the two double triplets at δ 6.61 (J=11.3 and 1.7 Hz) and at δ 5.46 (J=11.3 and 1.7 Hz) for the two =C<u>H</u>. The singlet at δ 4.56 is the -OC<u>H</u>$_2$-Ar-, finally and the doublet doublet at δ 4.35 (J=1.7 and 4.3 Hz) represents the -C<u>H</u>$_2$O-.

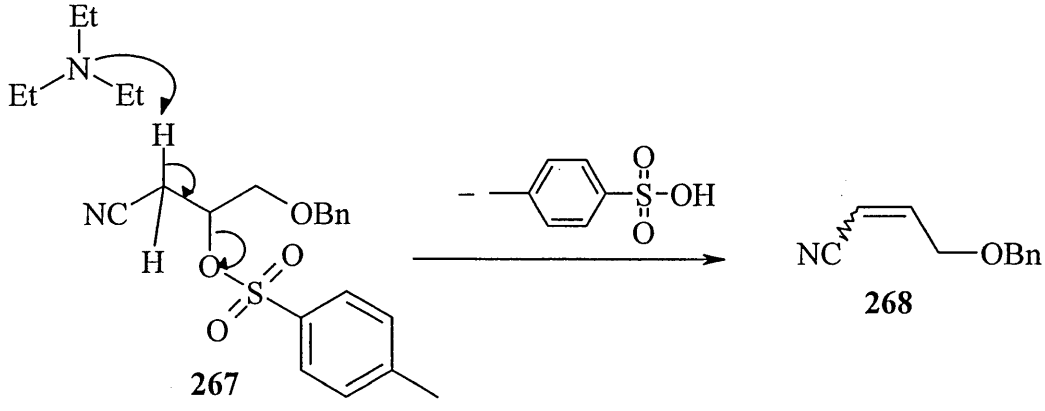


267

E : *Z* **268**
1.5 : 1

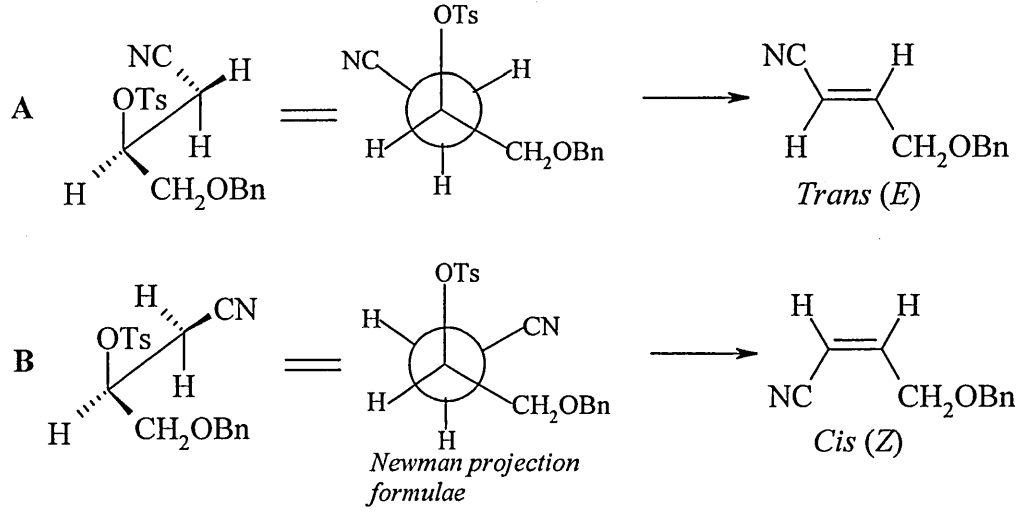
Scheme 182

In the reaction, the triethylamine attacks the most accessible hydrogen next to the nitrile given that the hydrogens close to the benzyl ether will be inaccessible due to steric hindrance. The elimination process follows the anti E2 mechanism, where the base attacks the proton that is anti periplanar to the leaving group of benzyl derivative **267**, to form the α,β-unsaturated nitrile **268** (scheme 183).



Scheme 183

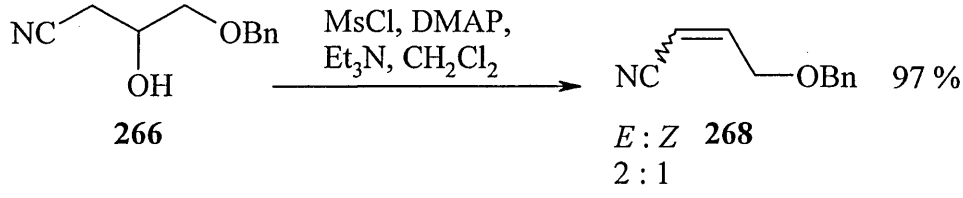
For benzyl derivative **267** two conformations (**A** and **B**) are possible for the transitional state (scheme 184). Conformation **A** does not have the eclipsing effect of **B** and therefore **A** is more stable and most of the elimination occurs from this conformation. Hence, the eclipsing effects, and factors such as the nature of the leaving group, the base, the solvent and the substrate all have considerable affect on the ratio of isomers.



Scheme 184

Since elimination of tosyl group from compound **267** facilitated by triethylamine was so effective, it was anticipated that similar reaction of 4-benzyloxy-

3-hydroxybutyronitrile **266** using methanesulphonyl chloride[249] and 4-dimethylaminopyridine with triethylamine in CH_2Cl_2 in one step would also produce the α,β-unsaturated nitrile **268**. The reaction afforded a brown oil in 97 % yield after column chromatography. Further purification by distilling a small fraction of the brown oil afforded a yellow oil. The IR and ^{1}HNMR analysis confirmed that the yellow oil is 4-benzyloxybut-2-enenitrile **268** (E and Z forms in the ratio of approximately 2:1) (scheme 185).

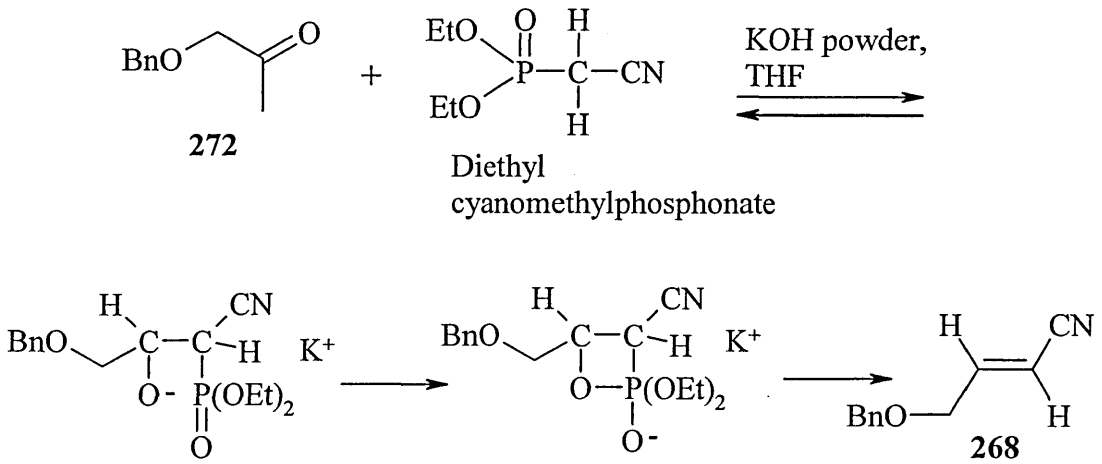


Scheme 185

The 4-benzyloxybut-2-enenitrile **268**, as the dienophile, now in hand should react with the 4-methyl-5-ethoxyoxazole in the Diels-Alder reaction to form the desired substituted pyridine, and therefore a sufficient amount of compound **268** is required. The synthesis of α,β-unsaturated nitrile **268** was conducted successfully in small scale. Scaling up the reaction caused purification problems, as repetitive distillation was required which resulted in a moderately low yield of reasonably pure α,β-unsaturated nitrile **268**. Therefore, the Wittig-Horner reaction was investigated in order to afford the 4-benzyloxybut-2-enenitrile **268** in a more efficient way.

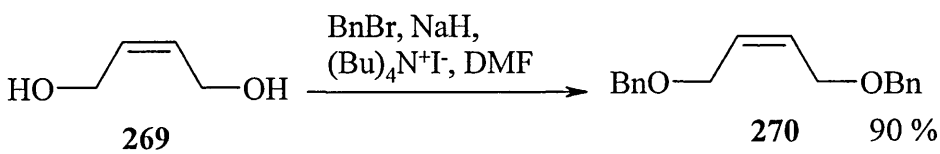
2.2.2.5. The Wittig-Horner reaction.

One of the most common methods in obtaining an alkene is through the reaction of phosphonate anions containing electron-withdrawing groups with aldehydes or ketones in an aprotic solvent. Direct conversion of the carbonyl compound to α,β-unsaturated nitrile, without isolating and dehydrating the intermediate β-hydroxy nitrile, is of considerable interest[251,252]. Therefore, benzyl protected α,β-unsaturated nitrile **268** can, in theory, be obtained by allowing 2-benzyloxyacetaldehyde **272** to react with the Wittig reagent, diethyl cyanomethylphosphonate, via the Wittig-Horner reaction (scheme 186).



Scheme 186

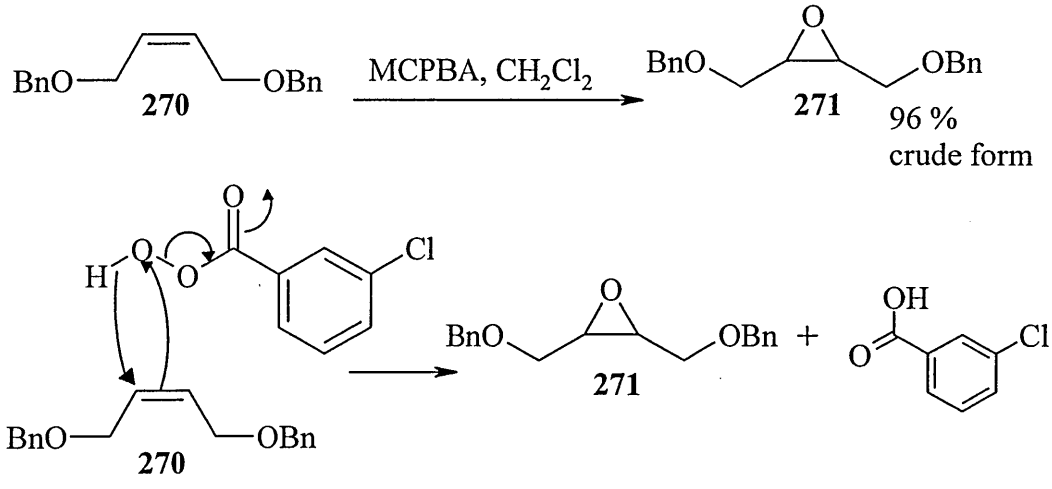
Preparation of the 2-benzyloxyacetaldehyde **272** began with the benzylation of 2-butene-1,4-diol **269** through the use of benzyl bromide with sodium hydride and catalytic amount of *tetra*-n-butylammonium iodide in DMF[250]. The reaction afforded a light yellow oil in 90 % yield after extracting the reaction mixture with ether/hexane and removal of the solvent. The analysis of the light yellow oil indicated the presence of the aromatic C-H stretch at 3030 cm^{-1}, the C-O bond at 1091 cm^{-1}, and the monosubstituted aromatic =C-H bend at 737 and 698 cm^{-1} on the IR spectrum. The ^{1}HNMR verified that the oil is 1,4-dibenzyloxy-2-butene **270** (scheme 187) by the mutiplet at δ 7.31 for the 10 H of the two phenyl groups, and the double triplet at δ 5.77 (J=3.7 and 1.0 Hz) for the HC=CH. The 4 H singlet at δ 4.46 is the two -OCH_2-Ar, and the double doublet at δ 4.03 represents the 4 hydrogens of the two -CH_2O-.



Scheme 187

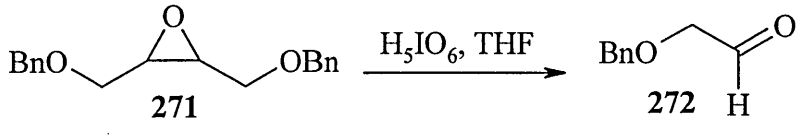
Without further purification, the obtained benzyl protected alkene **270** was subjected to an epoxidation reaction. The compound **270** was treated with *m*-chloroperbenzoic acid[250] in CH_2Cl_2 to afford an oily white solid after extracting the reaction mixture with ether and removal of solvent. The IR analysis of the solid revealed the aromatic C-H stretch at 3064 and 3031 cm^{-1}, the C-O bond at 1096 cm^{-1}, and the monosubstituted aromatic =C-H bend at 738 and 698 cm^{-1}. However, the peak for the C=C bond was no longer present. Additionally, the ^{1}HNMR showed the

10 H mutiplet at δ 7.29-7.38 to be the two phenyl groups, and the doublet at δ 4.61 (J=11.9 Hz) and at δ 4.51 (J=11.9 Hz) to be the two -OC<u>H</u>$_2$-Ar. The double doublet at δ 3.69 (J=3.9 and 11.3 Hz) and at δ 3.53 (J=6.5 and 11.3 Hz) to be the two -C<u>H</u>$_2$O-, and the multiplet at δ 3.26 to be the -C<u>H</u>-C<u>H</u>-. The ^{1}HNMR analysis confirmed that the solid is 1,4-benzyloxy-2,3-epoxybutane **271**. The reaction proceeds by the *m*-chloroperbenzoic acid transferring an oxygen atom to the alkene **270** in a cyclic, single step mechanism. The result is the syn addition of the oxygen to the alkene, with the formation of epoxide **271** and *m*-chlorobenzoic acid shown in scheme 188.



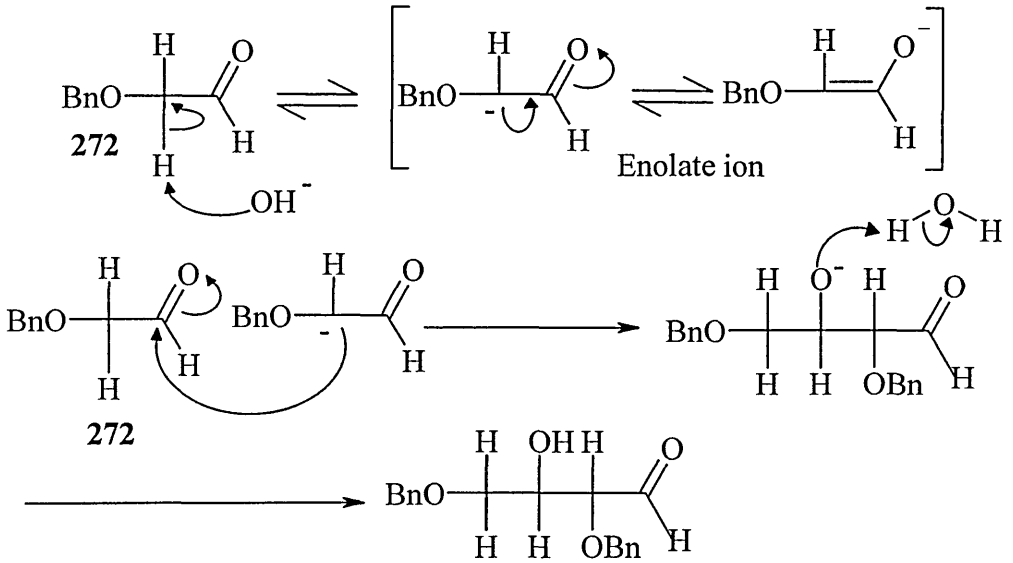
Scheme 188

Oxidative cleavage of epoxide **271** should give the desired 2-benzyloxyacetaldehyde **272**. Therefore, epoxide **271** was treated with periodic acid[250] to afford a yellow semi-solid crude after extracting with ether and removal of the solvent. The IR analysis of the crude revealed the C-H stretch at 2924 cm^{-1}, the C=O bond at 1727 cm^{-1} of the aldehyde, the C-O bond at 1095 cm^{-1}, and the monosubstituted aromatic =C-H bend at 736 and 699 cm^{-1}. The IR spectrum of the crude was adequate to indicate the presence of 2-benzyloxyacetaldehyde **272** (scheme 189).



Scheme 189

Carbonyl derivative **272** is a very sensitive compound, where deprotonation of the acidic α-hydrogen can easily occur forming the resonance-stabilised enolate ion which is readily capable of aldol condensation (scheme 190).



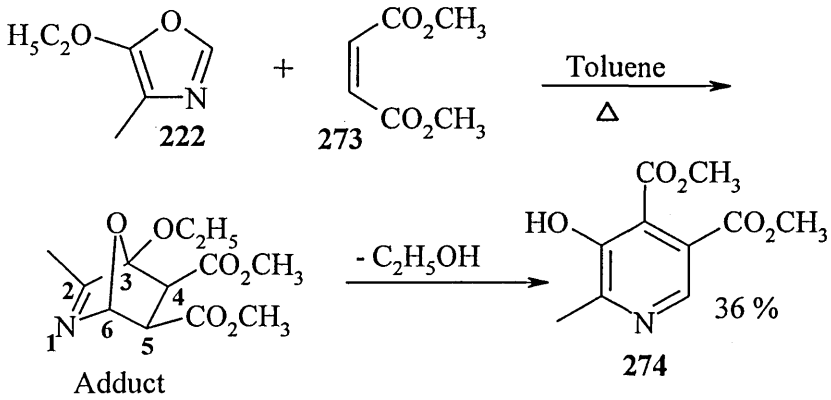
Scheme 190

Therefore, to minimise the formation of an aldol derivative the carbonyl derivative **272** was subjected to the Wittig-Horner reaction[252] immediately without further purification. The carbonyl compound was treated with diethyl cyanomethylphosphonate and powdered potassium hydroxide in THF to afford a dark residue after extracting with ether and removal of the solvent. The IR spectrum of the residue showed no sign of the nitrile group. Another attempt in the Wittig-Horner reaction failed to produce the desired benzyl protected α,β-unsaturated nitrile. It had been hoped that the presence of the ether oxygen would render **272** relatively stable towards base-catalysed aldol reactions. However, it appears that the reaction conditions were probably not optimum for α,β-unsaturated nitrile formation and the starting materials may have aldolised during the attempted reaction.

2.2.3. The Diels-Alder reaction.

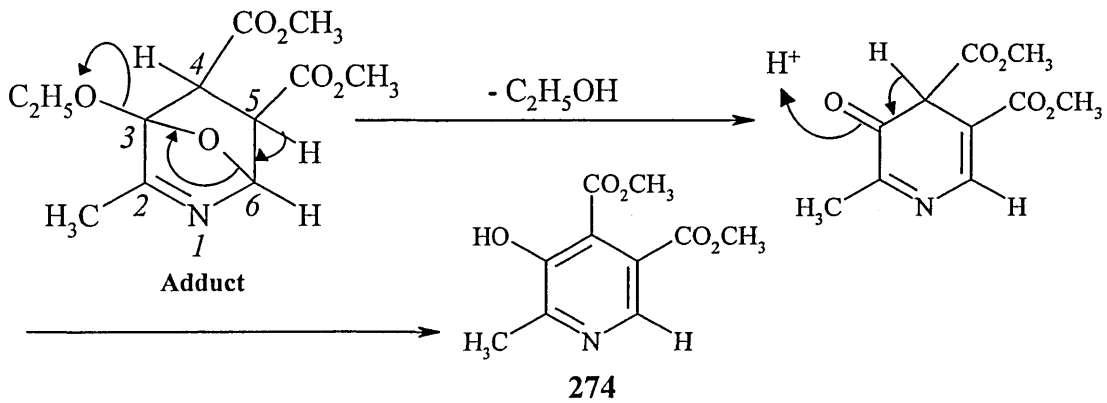
Although problems were associated with the synthesis of the desired dienophile, the satisfactory amount of 4-methyl-5-ethoxyoxazole **222** that had been synthesised was used in Diels-Alder cycloaddition with commercially available dienophiles as model reactions. 4-Methyl-5-ethoxyoxazole **222** was refluxed with dimethyl maleate **273** in toluene[253] to produce a cream solid in 36 % yield after column chromatography. The IR spectrum of the cream solid revealed the hydroxyl group at 3358 cm^{-1}, the C-H stretch at 2956 cm^{-1}, the C=O bond at 1743 cm^{-1}, and

the C-H bend at 1440 cm^{-1} in the IR spectrum. The ^{1}HNMR confirmed that the solid is dimethyl 5-hydroxypyridine-3,4-dicarboxylate **274** (scheme 191) having a singlet at δ 8.26 for the py-H, a singlet at δ 3.94 for the $-CO_2CH_3$, a singlet at δ 3.90 for the $-CO_2CH_3$, and a singlet at δ 2.57 for the py-CH_3.



Scheme 191

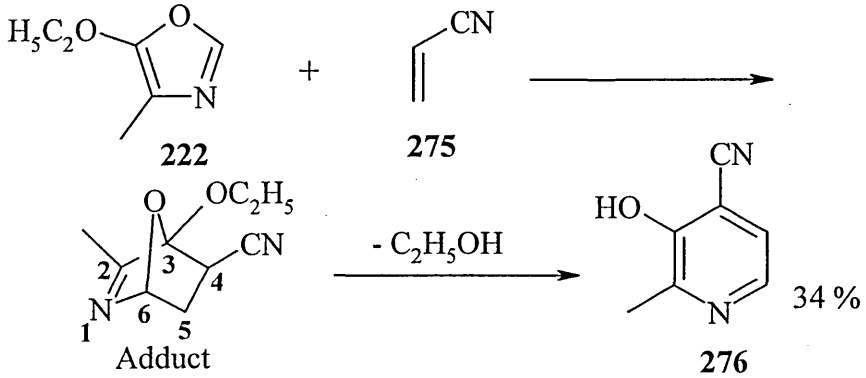
The oxazole **222** and the dienophile **273** react with each other in a concerted addition in which both of the new single bonds are formed at the same time. The formation of the two new σ-bonds occurs by the overlap of molecular π-orbitals in a direction corresponding to endwise overlap of atomic *p*-orbitals. The concerted addition gives the adduct. The dissociation of the C^6-O bond facilitated the elimination of the $-OC_2H_5$ group in the 3-position of the adduct and subsequent rearrangement forms the corresponding pyridine **274** (scheme 192).



Scheme 192

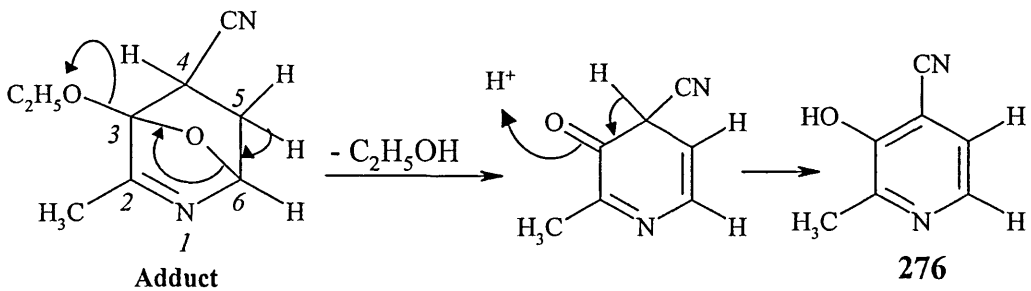
Another Diels-Alder reaction involved 4-methyl-5-ethoxyoxazole **222** with excess acrylonitrile[254] **275**. The reaction mixture was stirred at room temperature for 24 h to afford a light orange solid in 34 % yield after crystallising the residue from methanol. The IR analysis of the solid revealed the nitrile peak at 2224 cm^{-1}.

Additionally the ^{1}HNMR verified that the solid is 4-cyano-3-hydroxy-2-methylpyridine **276** (scheme 193) with a doublet at δ 8.00 (J=5 Hz) for the py-H, a doublet at δ 7.48 (J=5 Hz) for the py-H, and a singlet at δ 2.45 being the py-CH_3.



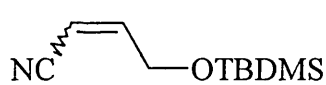
Scheme 193

A general rule for predicting the orientation of the cycloaddition is that the most electronegative substituent on the dienophile, in this case the –CN, will occupy the 4-position of the adduct. Hence, dissociation of the C^6-O bond facilitated the elimination of the -OC_2H_5 group (leaving group) in the 3-position of the adduct to form the desired pyridine **276** (scheme 194).



Scheme 194

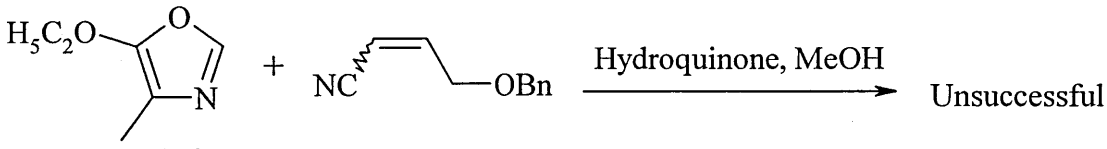
Thus, the prepared 4-methyl-5-ethoxyoxazole **222** undergoes Diels-Alder reaction reasonably effectively with simple, classic, commercially available dienophiles. Unfortunately, the yield of the prepared dienophile 4-(*tert*-butyldimethylsilanyloxy)but-2-enenitrile **261** was insufficient to be used in the Diels-Alder reaction with oxazole **222**.



261

Furthermore, attempted cycloaddition of 4-methyl-5-ethoxyoxazole **222** with the prepared 4-benzyloxybut-2-enetnitrile **268** was unsuccessful (scheme 195)[232]. The

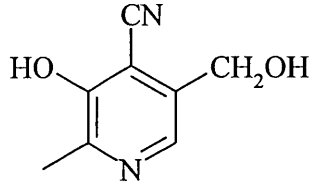
reaction was refluxed in methanol for 24 h and stored in the fridge for 24 h. Subsequent addition of ethanol saturated with hydrogen chloride gas to the reaction mixture did not produce any precipitate. In addition, the tlc analysis of the reaction mixture still showed the presence of the oxazole and the reaction was abandoned.



222 **268**

Scheme 195

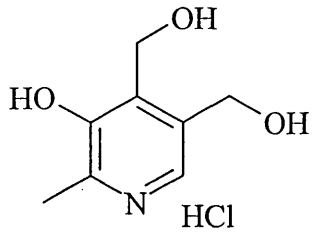
Repeated attempts with the conditions slightly varied also failed to give the desired pyridine **224** with benzyl protection at C5-hydroxymethyl.



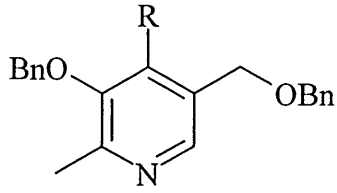
224

3. Conclusion

The diversity of manipulating commercially available pyridoxine hydrochloride **1** enables many vitamin B_6 analogues to be synthesised.



1



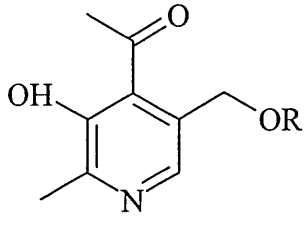
127 R = -CH_2OH

128 R = -CHO

241 R = —C(OH)—CH_3

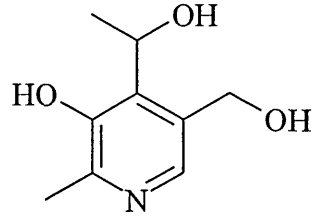
242 R = —C(=O)—CH_3

In this study, 3,5'-*O*-dibenzylpyridoxine **127** allowed selective modification of the 4-position to be conducted. The desired derivative **127** was obtained through multiple steps. Modifying the derivative **127** by oxidation has transformed the 4-position hydroxyl to an aldehyde group (compound **128**) in high yield, without interfering with neighbouring groups. Subsequent Grignard reaction and hydrolysis afforded the derivative **241** in yield as high as 85 %. Furthermore, the alcohol derivative **241** was subjected to oxidation to give the corresponding derivative **242** in high yield.



234 R = H

235 R = PO_3H_2

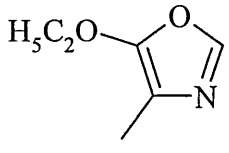


243

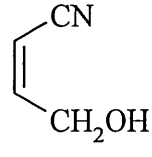
Attempts to achieve the desired pyridoxal analogue **234** and its phosphate form **235** by removing the benzyl groups were problematic. Pyridoxal and its derivatives have the tendency to exist in its cyclic hemiacetal form and the fact that vitamin B6 are highly soluble in water have made the task of isolating the desired derivative **234** difficult. Hence, repeated attempts in achieving the pyridoxal derivative **234** were unproductive. However, the attempt of removing the benzyl groups of compound **241** afforded pyridoxine derivative **243** in 75 % yield and since the pyridoxine and its derivatives does not have the problem with cyclic hemiacetal forms, the pyridoxine derivative **243** were easier to isolate. Attempts at phosphorylating the pyridoxine derivative **243** failed to achieve the desired phosphate

form and further progress on phosphorylation was abandoned due to the shortage of starting material.

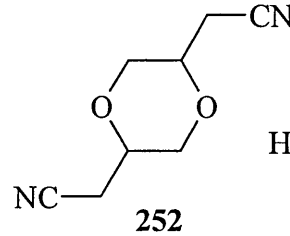
The Diels-Alder reaction of oxazole **222** with dienophile, α,β-unsaturated nitrile **223**, was another approach adopted for preparing vitamin B_6 analogues. The Robinson-Gabriel cyclodehydration of α-acylamino carbonyl compound produced the oxazole **222** in good yield. However, difficulties were associated with synthesising the α,β-unsaturated nitrile **223**. The α,β-unsaturated nitrile **223** is an extremely reactive and unstable compound that has the tendency to convert to its by-products, 2:5-biscyanomethyl 1:4-dioxan **252** and 2-aminofuran **253**.



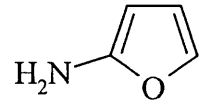
222



223

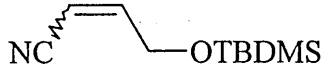


252

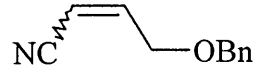


253

Therefore, addition of protective groups to the β-hydroxy nitrile prior to the formation of α,β-unsaturated nitriles was investigated and did produce a more stable derivative in the form of 4-(*tert*-butyldimethylsilanyloxy)but-2-enenitrile **261** and 4-benzyloxybut-2-enenitrile **268**. In addition, alternative approach in the attempts to form the protected α,β-unsaturated nitrile via the Wittig-Horner reaction was investigated, however, the reaction were unsuccessful.

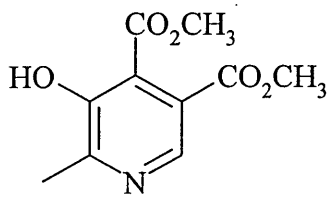


261

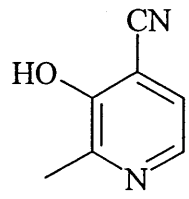


268

The Diels-Alder reaction of oxazole **222** with commercially available dienophiles, dimethyl maleate and acrylonitrile, was attempted as model reactions. The model reactions afforded dimethyl 5-hydroxypyridine-3,4-dicarboxylate **274** in 36% yield and 4-cyano-3-hydroxy-2-methylpyridine **276** in 34 % yield. However, Diels-Alder reaction of oxazole **222** with dienophile **268** failed to give the desired pyridine as tlc analysis showed only the presence of oxazole and repeated attempts with the condition slightly varied were unsuccessful. Since the starting materials, oxazole and dienophile, are lacking further progress on Diels-Alder reaction was discontinued.

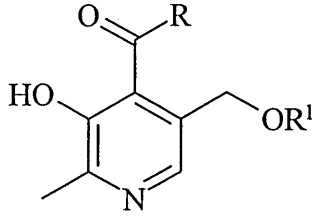


274



276

Due to the lack of starting materials, further progress in the two approaches to achieve the desired vitamin B_6 analogues **220** was discontinued. The desired vitamin B_6 analogues **220** could be use to examine biological systems such as the enzymatic binding sites of the mammalian multifunctional polypeptide CAD that is involved in the pyrimidine biosynthesis in mammalian cells. The biosynthesis of pyrimidine is essential for most growing cells and analogues could provide information on the binding sites in the mammalian multifunctional polypeptide CAD for the development of antiproliferative agents.



220

R = CH_3, C_2H_5 or longer chain.
R^1 = H or PO_3H_2

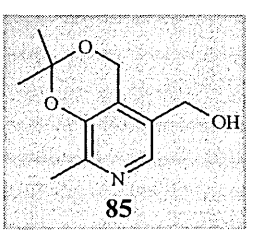
4. Experimental

Commercially available reagents were used throughout without further purification. Dried solvents were prepared by standard procedure. The drying agent used in the routine work up was anhydrous magnesium sulphate, unless stated otherwise. Analytical thin layer chromatography was carried out using Merck Kieselgel 60 F_{254} aluminium-backed plates, which were visualised either under ultra-violet light or with iodine vapour. Column chromatography was accomplished using pressurised short path columns with Merck Kieselgel 60H silica, eluted with stated solvents. Melting points were determined using an Electrothermal melting point apparatus and are uncorrected. IR spectra were recorded in the range 4000-500 cm^{-1} using a Perkin-Elmer Paragon 1600 FT-IR spectrometer. ^{1}HNMR spectra were recorded using a Bruker DPX-300 at 300MHz or a Jeol JMN PMX60si spectrometer at 60MHz.

4.1. Modification of pyridoxine.

Isopropylidenylation of pyridoxine hydrochloride.[132]

Dry acetone (100 mL) was saturated with hydrogen chloride gas, with cooling on an ice-bath to 0 °C, until the clear solvent turned yellow. Pyridoxine hydrochloride **1** (5 g, 24.31 mmol) was added to the stirred acetone, with a continuous flow of hydrogen chloride gas until the mixture turned golden yellow. The flask was sealed with a glass stopper and the mixture was stirred at room temperature for 75 min. The mixture was then placed in the fridge for 2 days. Ether (150 mL) was added to the chilled suspension, the white solid formed was filtered and converted to the free base by mixing it with excess saturated $NaHCO_3$. The solid was filtered and washed with cold water until the pH of washings were about 6. The white solid was dried in a vacuum oven at 50 °C, then in a vacuum desiccator over $CaCl_2$, to afford 3,4′-*O*-isopropylidenepyridoxine **85** (4.68 g, 92 %) as a white solid with mp 99-103 °C (lit. mp 110-111 °C[1]).



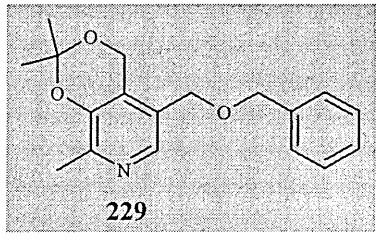
85

IR (KBr) ν 3106, 1414, 1054 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 1.55 (6H, s, isopropylidene CH_3), 2.37 (3H, s, 2-CH_3), 4.55 (2H, s, 4-CH_2OC-), 4.94 (2H, s, 5-CH_2OH), 7.82 (1H, s, 6-H).

Benzylation of 3,4'-*O*-isopropylidenepyridoxine.[1]

Dry DMF (100 mL) was added to sodium hydride (1.38 g, 57.36 mmol) and *tetra*-n-butylammonium iodide (0.1 g) at 0 °C under a N_2 atmosphere. 3,4'-*O*-Isopropylidenepyridoxine **85** (3 g, 14.34 mmol) in dry DMF (20 mL) was added to the suspended mixture. The reaction mixture was gradually heated to 65 °C and then cooled to 45 °C over a 90 min period. The mixture was cooled on ice to 0 °C and benzyl chloride (3.63 g, 28.68 mmol) was added dropwise and stirred overnight at room temperature. Water was added to the mixture, followed by extraction with ethyl acetate. The combined extracts were washed with water and dried. The solvent was removed *in vacuo* and the residue subjected to column chromatography (10-20 % ethyl acetate/hexane) to afford 3,4'-*O*-isopropylidene-5'-*O*-benzylpyridoxine **229** (3.06 g, 71 %) as a yellow oil.



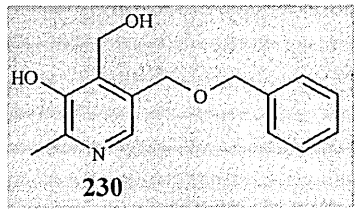
229

IR (neat) ν 2942, 1604, 1454, 1143, 739, 699 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 1.52 (6H, s, isopropylidene C<u>H</u>$_3$), 2.41 (3H, s, 2-C<u>H</u>$_3$), 4.43 (2H, s, -OC<u>H</u>$_2$-Ar), 4.49 (2H, s, 4-C<u>H</u>$_2$OC-), 4.87 (2H, s, 5-C<u>H</u>$_2$OBn), 7.22-7.42 (5H, m, Ar-<u>H</u>), 7.98 (1H, s, 6-<u>H</u>).

Acidic cleavage of 3,4'-*O*-isopropylidene-5'-*O*-benzylpyridoxine with formic acid.[216]

Formic acid, 98 % (10 mL) was added to a solution of 3,4'-*O*-isopropylidene-5'-*O*-benzylpyridoxine **229** (5 g, 16.70 mmol) in distilled water (10 mL). The mixture was stirred at 50 °C for 24 h. The mixture was adjusted to neutral with saturated $NaHCO_3$, extracted several times with CH_2Cl_2 (30 mL). The organic extracts were dried and concentrated *in vacuo*, followed by column chromatography (70 % ethyl acetate/petrol) to afford 5'-*O*-benzylpyridoxine **230** (2.91 g, 67 %) as a yellow solid with mp 112-114 °C (lit. mp 117-118 °C[1]).



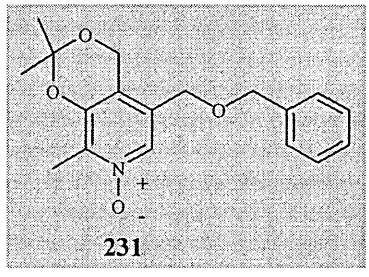
230

IR (KBr) ν 3032, 1420, 1121.6, 728.2 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 2.42 (3H, s, 2-C$\underline{H_3}$), 4.40 (2H, s, -OC$\underline{H_2}$-Ar), 4.45 (2H, s, 4-C$\underline{H_2}$OH), 5.00 (2H, s, 5-C$\underline{H_2}$OBn), 7.20-7.40 (5H, m, Ar-$\underline{H}$), 7.79 (1H, s, 6-$\underline{H}$), 8.40 (1H, br s, 3-O$\underline{H}$).

N-Oxidation of 3,4′-*O*-isopropylidene-5′-*O*-benzylpyridoxine.[217]

A solution of *m*-chloroperbenzoic acid (2.77 g, 16.04 mmol, 50 % pure) in $CHCl_3$ (50 mL) was added to a stirred solution of 3,4′-*O*-isopropylidene-5′-*O*-benzylpyridoxine **229** (2 g, 6.68 mmol) in $CHCl_3$ (25 mL) over a 10 min period. The reaction mixture was left stirring at room temperature for 1 h, diluted with $CHCl_3$ and shaken with 10 % sodium metabisulphite solution (to destroy the peracid) followed by saturated $NaHCO_3$. The organic extracts were washed with brine, water, and dried. The solvent was removed *in vacuo* to give crude 3,4′-*O*-isopropylidene-5′-*O*-benzylpyridoxine N-oxide **231** (2.02 g, 96 %) as a partly crystallising brown solid.



231

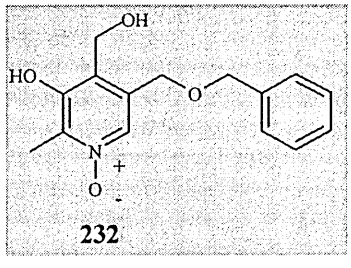
A small amount of the crude product was re-crystallised from acetone to give a white solid for spectroscopic analysis, mp 151-153 °C.

IR (KBr) ν 2997, 1451, 1209, 1097, 754, 700.0 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 1.58 (6H, s, isopropylidene C$\underline{H_3}$), 2.50 (3H, s, 2-C$\underline{H_3}$), 4.47 (2H, s, -OC$\underline{H_2}$-Ar), 4.56 (2H, s, 4-C$\underline{H_2}$OC-), 4.87 (2H, s, 5-C$\underline{H_2}$OBn), 7.22-7.44 (5H, m, Ar-$\underline{H}$), 8.18 (1H, s, 6-$\underline{H}$).

Acidic cleavage of 3,4′-*O*-isopropylidene-5′-*O*-benzylpyridoxine N-oxide with formic acid.[216]

Formic acid, 98 % (15 mL) was added to a solution of 3,4′-*O*-isopropylidene-5′-*O*-benzylpyridoxine N-oxide **231** (2 g, 6.34 mmol) in distilled water (15 mL). The solution was stirred at 50 °C for 2 days. The solution was then adjusted to neutral with saturated $NaHCO_3$, and the white solid formed was filtered, then dried in vacuum oven at 50 °C, to give 5′-*O*-benzylpyridoxine N-oxide **232** (0.2 g, 11 %) as a white solid with mp 116-125 °C.



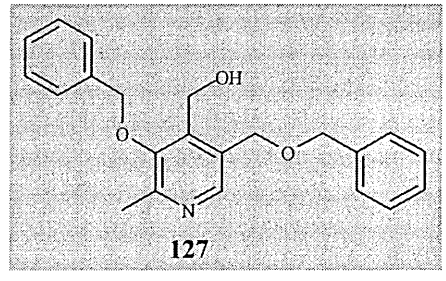
232

IR (KBr) ν 3030, 1364, 1109, 741 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 2.38 (3H, s, 2-C$\underline{H_3}$), 4.20 (2H, s, -OC$\underline{H_2}$-Ar), 4.49 (2H, s, 4-C$\underline{H_2}$OH), 4.76 (2H, s, 5-C$\underline{H_2}$OBn), 7.66 (1H, s, 6-$\underline{H}$), 7.00-7.49 (5H, m, Ar-$\underline{H}$), 9.80 (1H, br s, 3-O$\underline{H}$).

Benzylation of 5′-*O*-benzylpyridoxine using reagent benzyldimethylphenyl-ammonium chloride.[216]

A solution of benzyldimethylphenylammonium chloride[218] (2.96 g, 11.96 mmol) in dry MeOH (25 mL) was added to a solution of anhydrous sodium methoxide (0.83 g, 15.43 mmol) in dry MeOH (25 mL) at 0 °C under N_2 atmosphere. A solution of 5′-*O*-benzylpyridoxine **230** (1.40 g, 5.40 mmol) in dry MeOH (25 mL) was added to the reaction mixture. After stirring for 2 h at room temperature, the resultant mixture was transferred to a separating funnel and was dripped, over a 30 min period, into hot toluene (100 mL). During the addition, gentle heat was provided so that the MeOH was slowly distilled via a condenser to a receiving flask. The mixture was cooled, and the excess toluene was evaporated under reduced pressure to leave a brown residue. The residue was dissolved in saturated NH_4Cl solution and extracted with CH_2Cl_2. The organic extracts were dried and concentrated *in vacuo*, followed by column chromatography (40-50 % ethyl acetate/petrol) to afford 3,5′-*O*-dibenzylpyridoxine **127** (1.61 g, 85 %) as a yellow solid with mp 62-66 °C (lit. mp 64-69 °C[1]).



127

IR (KBr) ν 3125, 3032, 2925, 1450, 1100, 744.4, 696.1 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 2.54 (3H, s, 2-C$\underline{H_3}$), 3.34 (1H, br s, -O$\underline{H}$), 4.62 (2H, s, -OC$\underline{H_2}$-Ar), 4.64 (2H, s, -OC$\underline{H_2}$-Ar), 4.71 (2H, s, 4-C$\underline{H_2}$OH), 4.98 (2H, s, 5-C$\underline{H_2}$OBn), 7.28-7.50 (10H, m, Ar-$\underline{H}$), 8.23 (1H, s, 6-$\underline{H}$).

Oxidation of 3,5′-*O*-dibenzylpyridoxine.

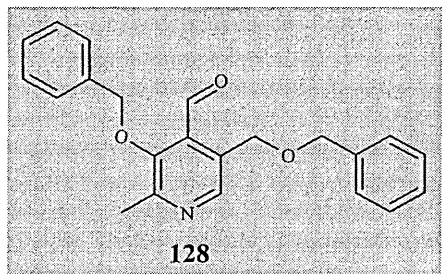
Oxidation of 3,5′-*O*-dibenzylpyridoxine with activated MnO_2.[217]

Activated MnO_2 (0.50 g, 5.71 mmol) in dry CH_2Cl_2 (40 mL) was added to a stirred and cooled (ice bath) solution of 3,5′-*O*-dibenzylpyridoxine **127** (0.5 g, 1.43 mmol) in dry CH_2Cl_2 (40 mL). The reaction mixture was stirred at room temperature

for 2 days. The mixture was filtered through a Celite pad and washed with CH_2Cl_2 (50 mL). The filtrate was concentrated *in vacuo* and the residue purified by column chromatography (ethyl acetate/petrol) to give 3,5′-*O*-dibenzylpyridoxal **128** (0.05 g, 10 %) as a light yellow solid.

Oxidation of 3,5′-*O*-dibenzylpyridoxine with pyridinium dichromate.[216]

Pyridinium dichromate (0.43 g, 1.14 mmol), anhydrous sodium acetate (0.02 g, 0.23 mmol) and powdered 3-Å molecular sieves (0.05 g) in dry CH_2Cl_2 (30 mL) was stirred at 0 °C under N_2 atmosphere. 3,5′-*O*-Dibenzylpyridoxine **127** (0.2 g, 0.57 mmol) in dry CH_2Cl_2 (10 mL) was added to the suspension. The reaction mixture was stirred at room temperature for 24 h and then an equal volume of anhydrous ether was added to the mixture and stirring continued for an additional of 30 min. The mixture was concentrated *in vacuo* and purified by column chromatography (ethyl acetate/petrol) to give 3,5′-*O*-dibenzylpyridoxal **128** (0.19 g, 96 %) as a light yellow solid.



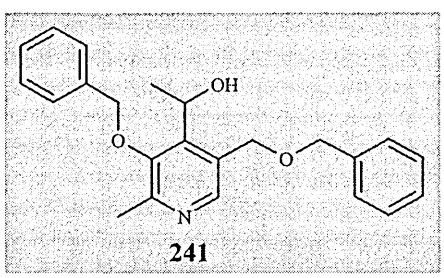
128

IR (KBr) ν 2874, 1697, 1362, 1062, 740, 698 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 2.62 (3H, s, 2-C<u>H</u>$_3$), 4.64 (2H, s, -OC<u>H</u>$_2$-Ar), 4.83 (2H, s, -OC<u>H</u>$_2$-Ar), 4.98 (2H, s, 5-C<u>H</u>$_2$OBn), 7.12-7.48 (10H, m, Ar-<u>H</u>), 8.65 (1H, s, 6-<u>H</u>), 10.37 (1H, s, 4-C<u>H</u>O).

<u>Grignard reaction of 3,5′-*O*-dibenzylpyridoxal with methylmagnesium bromide.</u>[223]

Methylmagnesium bromide (5 mL, 15.50 mmol, of a 3 M solution in Et_2O) was added to a cooled and stirred suspension of 3,5′-*O*-dibenzylpyridoxal **128** (3 g, 8.64 mmol) in anhydrous Et_2O (100 mL) under N_2 atmosphere. The grey mixture was stirred for a further 4 h at room temperature. Then ice-cold saturated NH_4Cl solution (25 mL) was added carefully and allowed to stand for 10 min. The reaction mixture was extracted with Et_2O and washed with water. The combined extracts were dried and solvent removed *in vacuo* to afford 4'-methyl-3,5'-*O*-dibenzylpyridoxine **241**



241

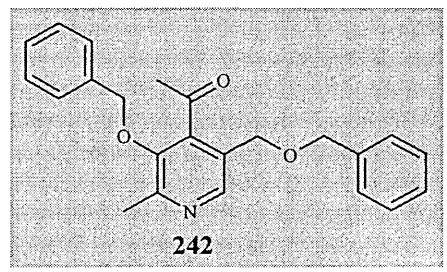
(2.68 g, 85 %) as a yellow oil.

IR (neat) ν 3246, 2927, 1454, 1069, 737, 697 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 1.53 (3H, d, J=6.7Hz, -C- C$\underline{H}_3$), 2.58 (3H, s, 2-C$\underline{H}_3$), 4.60 (2H, s, -OC$\underline{H}_2$-Ar), 4.78 (2H, s, -OC$\underline{H}_2$-Ar), 4.92 (2H, s, 5-C$\underline{H}_2$OBn), 5.27 (1H, q, J=6.7Hz, 4-C$\underline{H}$OH), 7.28-7.45 (10H, m, Ar-$\underline{H}$), 8.20 (1H, s, 6-$\underline{H}$).

Oxidation of 4'-methyl-3,5'-*O*-dibenzylpyridoxine with pyridinium dichromate.[216]

Pyridinium dichromate (2.48 g, 6.6 mmol), anhydrous sodium acetate (0.16 g, 1.98 mmol) and powdered 3-Å molecular sieves (0.05 g) in dry CH_2Cl_2 (40 mL) was stirred at 0 °C under N_2 atmosphere. 4'-Methyl-3,5'-*O*-dibenzylpyridoxine **241** (1.2 g, 3.3 mmol) in dry CH_2Cl_2 (10 mL) was added to the suspension. The reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated *in vacuo* and the residue purified by column chromatography (ethyl acetate/petrol) to afford 4'-methyl-3,5'-*O*-dibenzylpyridoxal **242** (1.15 g, 96 %) as a yellow oil.



242

IR (neat) ν 3032, 2925, 1706, 1353, 1091, 738, 699 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 2.47 (3H, s, 2-C$\underline{H}_3$), 2.53 (3H, s, 4-COC$\underline{H}_3$), 4.51 (4H, s, -OC$\underline{H}_2$-Ar), 4.81 (2H, s, 5-C$\underline{H}_2$OBn), 7.29-7.40 (10H, m, Ar-$\underline{H}$), 8.25 (1H, s, 6-$\underline{H}$).

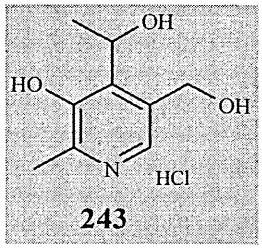
Attempted reaction of 4'-methyl-3,5'-*O*-dibenzylpyridoxal with trifluoroacetic acid.[148]

4'-Methyl-3,5'-*O*-dibenzylpyridoxal **242** (0.46 g, 1.27 mmol) and trifluoroacetic acid (15 mL) was stirred at room temperature for 24 h and then refluxed for 10 h. The excess trifluoroacetic acid was removed *in vacuo* and the residue dissolved into a small amount of water, which was passed through an Amberlite CG-50 column eluting with water. The effluent fractions with similar UV absorption were combined and the water was removed *in vacuo*. The oily residue was taken up in a small amount of EtOH. Precipitation did not occur when Et_2O containing HCl (g) was added to the mixture, but it remained as a thick oily residue.

The IR spectrum of the residue remained ambiguous, therefore, the reaction abandoned.

Reaction of 4'-methyl-3,5'-*O*-dibenzylpyridoxine with trifluoroacetic acid.[148]

4'-Methyl-3,5'-*O*-dibenzylpyridoxine **241** (1.07 g, 2.94 mmol) and trifluoroacetic acid (20 mL) was stirred at room temperature for 24 h and then refluxed for 10 h. The excess trifluoroacetic acid was removed *in vacuo* and the residue dissolved into a small amount of water, which was passed through an Amberlite CG-50 column eluting with water. The effluent fractions with similar UV absorption were combined and the water was removed *in vacuo*. The oily residue was taken up in a small amount of EtOH. 4'-Methylpyridoxine **243** (0.48 g, 75 %) with mp 176-177 °C (lit. mp 177-178 °C[223]) was obtained as a cream coloured hydrochloride salt on the addition of Et_2O containing HCl (g). .



243

IR (KBr) ν 3326, 2968, 1394 cm^{-1}.

^{1}HNMR (300MHz, DMSO-d_6) δ 1.43 (3H, d, J=6.7 Hz, -CH-C<u>H</u>$_3$), 2.58 (3H, s, 2-C<u>H</u>$_3$), 4.69 (2H, s, 5-C<u>H</u>$_2$-), 5.38 (1H, q, J=6.7 Hz, 4-C<u>H</u>-), 8.16 (1H, s, 6-<u>H</u>).

Attempted phosphorylation of 4'-methylpyridoxine.[24]

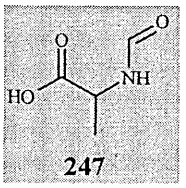
4'-Methylpyridoxine **243** (0.2 g, 0.91 mmol) and a phosphorylating mixture (1 mL), consisting of 1 part of P_2O_5 and 1.3 part of H_3PO_4 (by weight), was stirred for 4 h in an oil bath at 60 °C. The mixture was passed through an Amberlite CG-50 column eluting with water. The collected fractions were combined and the water was removed by freeze-drying to give a small amount of crystals. The IR spectrum of the crystals was ambiguous, therefore the reaction abandoned.

4.2. Total syntheses of vitamin B_6 analogues.

N-Formylation of (±)-alanine.[225]

A mixture of 98% formic acid (30 mL) and acetic anhydride (60 mL) was heated at a temperature of 50-60 °C for 2 h in a dry atmosphere of N_2. The mixture was cooled to room temperature and (±)-alanine **246** (8.9 g, 0.1 mol) in formic acid (20 mL) was added gradually over a period of 15 min with the temperature being

maintained below 35 °C and stirred was for a further 40 min. The temperature was raised to room temperature and stirred for a further 80 min. Cold water (80 mL) was added to the mixture and it was concentrated *in vacuo*. Crystallisation using ethyl acetate/ethanol gives N-formyl-(±)-alanine **247** (6.88 g, 59%) as a white solid with mp 142-147 °C (lit. mp 145-148 °C[226]).

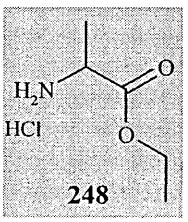


247

IR (nujol) ν 3355, 2924, 2854, 1702 cm^{-1}.

Esterification of (±)-alanine.[228]

Thionyl chloride (35.7 g, 0.3 mol) was added dropwise to a cooled, stirred suspension of (±)-alanine **246** (17.8 g, 0.2 mol) in 95 % EtOH (150 mL), at such a rate that the reaction mixture refluxes lightly. The mixture was refluxed for 2 h, then the solvent evaporated *in vacuo* and the residue dissolved in EtOH (100 mL). Ether was added to precipitate the hydrochloride, which was collected by filtration, washed with ether and dried over $CaCl_2$ to give (±)-alanine ethyl ester hydrochloride **248** (30 g, 98 %) as a white solid, mp 78-82 °C (lit. mp 87-88 °C[229]).



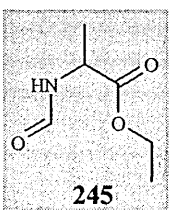
248

IR (nujol) ν 2924, 2854, 1746, 1462, 1377 cm^{-1}.

Synthesis of N-formyl-(±)-alanine ethyl ester.

Esterification of N-formyl-(±)-alanine using ethanol under acidic condition.[227]

N-Formyl-(±)-alanine **247** (3g, 25.6 mmol) in 95% EtOH (30 mL) with 3-5 drops of concentrated H_2SO_4 was refluxed gently for 6 h. The reaction mixture was cooled and EtOH was removed *in vauco*. The residue was dissolved in water and extracted with ethyl acetate. The combined extracts were washed with water, brine, saturated $NaHCO_3$, and dried. The solvent was evaporated *in vacuo* and residue distilled at 110 °C / 3 mm Hg (lit. bp 100 °C / 1 mm Hg[226]) to give N-formyl-(±)-alanine ethyl ester **245** (1.11 g, 30 %) as a clear oil.



245

IR (neat) ν 3300, 2986, 1740,1205 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 1.30 (3H, t, $C\underline{H}_3CH_2$-), 1.45 (3H, d, $C\underline{H}_3CH$-), 4.22 (2H, q, -$C\underline{H}_2$-), 4.65 (1H, q, -$C\underline{H}$-), 7.08 (1H, br, -$N\underline{H}$-), 8.20 (1H, s, -$C\underline{H}O$).

N-formylation of (±)-alanine ethyl ester hydrochloride with a mixture of formic acid and acetic anhydride.[225]

A mixture of 98% formic acid (1.5 mL) and acetic anhydride (3 mL) was heated at a temperature of 50-60 °C for 2 h in dry atmosphere. (±)-Alanine ethyl ester hydrochloride **248** (1 g, 6.5 mmol) in formic acid (1.5 mL) was added gradually to the mixture over a period of 15 min with temperature maintained below 35 °C and stirred for a further 3 h. The mixture was concentrated *in vacuo* and residue distilled at 110 °C / 3 mm Hg to give a very small amount of clear oil (0.4 g), which the tlc and IR spectrum indicated to be N-formyl-(±)-alanine ethyl ester **245**.

N-formylation of (±)-alanine ethyl ester hydrochloride with triethylamine and p-toluenesulphonic acid monohydrate.[230]

Triethylamine (1.11g, 0.11 mol) was added to a stirred mixture of (±)-alanine ethyl ester hydrochloride **248** (15.4 g, 0.1 mol) and *p*-toluenesulphonic acid monohydrate (0.01 g, 0.05 mmol) in ethyl formate (50 mL). The mixture was refluxed for 20 h and then cooled to room temperature. The triethylamine hydrochloride salt was removed by filtration and filtrate concentrated to 15 mL. On cooling to -5°C, more triethylamine hydrochloride salt formed and was removed. Distillation of the filtrate at 110 °C / 3 mm Hg afforded a very small amount of clear oil (0.2 g) and the IR spectrum indicated that it was N-formyl-(±)-alanine ethyl ester **245**.

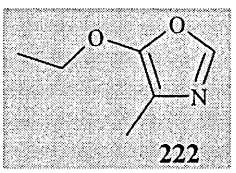
N-formylation of (±)-alanine ethyl ester hydrochloride with formamide.[231]

A mixture of (±)-alanine ethyl ester hydrochloride **248** (15 g, 95.7 mmol) and formamide (4.2 g, 3.7 mL, 93.3 mmol) was heated slowly to 105 °C over a period of 45 min. After heating at 105 °C for 10 min, toluene (30 mL) was added, the mixture was kept at room temperature overnight and then refluxed for 6 h. The solution was cooled and the NH_4Cl formed was removed by filtration and the filtrate collected. The filtrate was concentrated to a residue and distilled at 110 °C / 3 mm Hg to give N-formyl-(±)-alanine ethyl ester **245** (11.87 g, 85 %) as a clear oil.

Synthesis of 4-methyl-5-ethoxyoxazole.

Robinson-Gabriel reaction of N-formyl-(±)-alanine ethyl ester with phosphorus pentoxide.[232]

To a 250 mL, 3-necked round-bottom flask equipped with a Hershberg stirrer and a reflux condenser was added N-formyl-(±)-alanine ethyl ester **245** (5 g, 34.5 mmol), phosphorus pentoxide (19.6 g, 137.9 mmol) and dry $CHCl_3$ (100 mL). The resulting suspension was refluxed in a dry N_2 atmosphere with vigorous stirring for 12 h. Then ice-cold 10 % $NaHCO_3$ solution (100 mL) was added, to decompose unreacted phosphorus pentoxide, and the pH was adjusted to neutral with concentrated NaOH. After extraction with CH_2Cl_2 and washing with brine and water, the organic extracts were dried, concentrated and purified by distillation at 75-80 °C / 50 mm Hg (lit. bp 78-80 °C / 50 mm Hg[232]) to afford 4-methyl-5-ethoxyoxazole **222** (2.36 g, 54 %) as a clear liquid.



222

IR (neat) ν 2929, 1336, 1221 cm^{-1}.

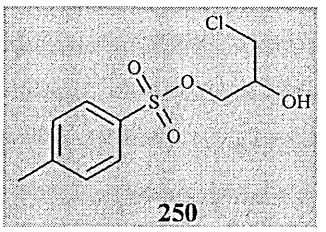
^{1}HNMR (300MHz, $CDCl_3$) δ 1.38 (3H, t, $C\underline{H}_3$-CH_2-), 2.04 (3H, s, $C\underline{H}_3$-), 4.12 (2H, q, -$C\underline{H}_2$O-), 7.40 (1H, s, -OC$\underline{H}$-).

Attempted cyclisation of N-formyl-(±)-alanine ethyl ester using triphenyl phosphine and iodine under basic conditions.[233]

A solution of N-formyl-(±)-alanine ethyl ester **245** (5.41 g, 37.29 mmol) in dry CH_2Cl_2 (10 mL) and triethylamine (15.47 g, 152.9 mmol) were added sequentially to a stirred solution of triphenyl phosphine (19.56 g, 74.57 mmol) and iodine (18.93 g, 74.58 mmol) in dry CH_2Cl_2 (40 mL) at room temperature under N_2 atmosphere. The reaction mixture was stirred for 24 h. The dark mixture was then washed with of saturated $Na_2S_2O_3$ solution (60mL) and subsequently extracted with ether. The organic phase was dried and solvent removed *in vacuo* leaving a dark residue. Vacuum distillation of the residue afforded clear oil, but the IR and ^{1}Hnmr spectra of the oil showed no trace of the desired oxazole.

Reaction of epichlorohydrin with p-toluenesulphonic acid.[234]

Epichlorohydrin **249** (25 g, 0.27 mol) was added dropwise over 30 min to a stirred solution of *p*-toluenesulphonic acid (47.5 g, 0.25 mol) in CH_2Cl_2 (200 mL) at 5-10 °C. The reaction mixture was refluxed for 3 h cooled and then the mixture was washed twice with saturated $NaHCO_3$ solution (200 mL), followed by extraction with CH_2Cl_2 and dried. The solvent was removed *in vacuo* and the residue purified by column chromatography (10-5:1 hexane/acetone) to give 1-chloro-3-(toluene-4-sulphonyl)propan-2-ol **250** (51.71 g, 72 %) as a yellow oil.



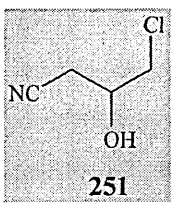
250

IR (neat) ν 3515, 2959, 1358, 815 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 2.45 (3H, s, C<u>H</u>$_3$-Ar), 2.80 (1H, d, -OH), 3.50-4.20 (5H, m, -C<u>H</u>$_2$C<u>H</u>(OH)C<u>H</u>$_2$-), 7.32, 7.80 (each 2H, d, Ar-<u>H</u>).

Reaction of 1-chloro-3-(toluene-4-sulphonyl)propan-2-ol with KCN in methanol.[235]

Potassium cyanide (16 g, 0.25 mol) was added to a mixture of 1-Chloro-3-(toluene-4-sulphonyl)propan-2-ol **250** (65 g, 0.25 mol) in MeOH (350 mL) and stirred for 40 h at room temperature. The potassium tosylate formed was removed by filtration and the product distilled at 115 °C / 4 mm Hg (lit. bp 134-136 °C / 13 mm Hg[221]) to give 3-chloro-2-hydroxybutyronitrile **251** (3.58 g, 12 %) as a clear yellow liquid.



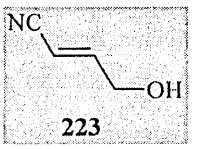
251

IR (neat) ν 3430, 2962, 2256, 1415 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 2.70 (2H, m, C<u>H</u>$_2$CN), 3.50 (1H, s, -O<u>H</u>), 3.65 (2H, d, C<u>H</u>$_2$Cl), 4.28 (1H, m, C<u>H</u>OH).

Reaction of 3-chloro-2-hydroxybutyronitrile with NaOH.[236]

3-Chloro-2-hydroxybutyronitrile **251** (1 g, 8.36 mmol) and NaOH (50 mL, 0.96 M) was stirred at room temperature for a period of 90 min. The reaction mixture was subjected to continuous extraction with ethyl acetate using a continuous extraction apparatus. The organic extracts were dried and concentrated *in vacuo*. The residue was distilled at 136-145 °C / 15 mm Hg (lit. bp 119-120 °C / 13 mm Hg[236]) to give 4-hydroxybut-2-enenitrile **223**



223

(0.21 g, 30 %) as a yellow liquid. The liquid partly crystallised after standing for several days at room temperature.

IR (neat) ν 3430, 2920, 2227, 1639 cm^{-1}.

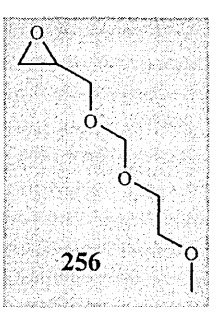
^{1}HNMR (300MHz, $CDCl_3$) E form δ 3.89 (1H, s, -OH), 4.20 (2H, s, -CH_2-), 5.68 (1H, d, J=16 Hz, =CH), 6.92 (1H, d, J=16 Hz, =CH).

Reaction of epichlorohydrin with alkali cyanides.[236]

A solution of epichlorohydrin **249** (30 g, 0.32 mol) in ethanol (65 mL) with a few drops of thymol-blue was stirred at room temperature. Sodium cyanide (8.3 g, 0.17 mol) and potassium cyanide (11.1 g, 0.17 mol) in water (75 mL) was added gradually to the solution over a period of 30 min with cooling. Neutrality was maintained throughout by the drop-wise addition of dilute acetic acid and for an additional 30 min after all the cyanide had been added. The solution was set aside for 1 h at 0 °C, and continuously extracted with ethyl acetate. The organic phase was dried and solvent removed *in vacuo* to afford a residue, which was vacuum distilled at 136-145 °C / 15 mm Hg to give 0.98 g of yellow liquid. The IR spectrum indicated a presence of a mixture of 3-chloro-2-hydroxybutyronitrile **251** and 4-hydroxybut-2-enenitrile **223**. The liquid partly crystallised after standing for several days, which indicate dimerisation have occurred.

Reaction of glycidol with methoxyethoxymethyl chloride.[238]

Glycidol **255** (4 g, 54 mmol) was added dropwise to a stirred mixture of sodium hydride (1.56 g, 64.80 mmol) in dry THF (50 mL) at 0 °C in a dry atmosphere. 2-Methoxyethoxymethyl chloride (8.07 g, 64.80 mmol) was added dropwise to the mixture at 0 °C and stirred for 15 min. The reaction mixture was stirred at room temperature for 2 h and left to stand overnight. Water was added to decompose excess sodium hydride and the mixture extracted with ethyl acetate. The combined extracts were washed with brine, followed by water, and dried. The solvent was removed *in vacuo* to give crude 2-(2-methoxyethoxymethoxy)oxirane **256** (3.24 g, 37 %) as a light yellow oil.

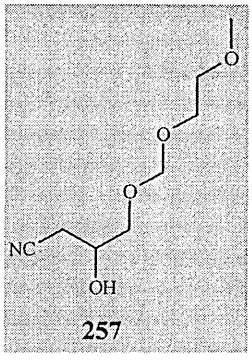


256

IR (neat) ν 2886, 1458, 1047 cm^{-1}.

Reaction of 2-(2-methoxyethoxymethoxy)oxirane with KCN.[239]

2-(2-Methoxyethoxymethoxy)oxirane **256** (3.24 g, 19.98 mmol) in CH_3CN (80 mL) was treated with anhydrous lithium perchlorate (3.19 g, 29.97 mmol) and potassium cyanide (2.08 g, 31.96 mmol). The reaction mixture was stirred overnight at 70 °C and then cooled to room temperature. The mixture was diluted with water and extracted with ether. The combined extracts were washed with saturated $NaHCO_3$, followed by water, and dried. The solvent was removed *in vacuo* to give crude 2-hydroxy-4-(2-methoxyethoxymethoxy)butyronitrile **257** (0.21 g, 5.6 %) as a yellow oil.



257

IR (neat) ν 3448, 2934, 2252, 1039 cm^{-1}.

Synthesis of 1-*O*-*tert*-butyldimethylsilyl-2,3-epoxypropane.

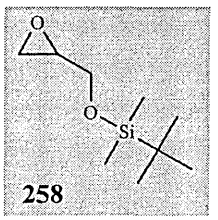
Attempted reaction of glycidol with *tert*-butyldimethylsilyl chloride and imidazole in DMF.[240]

Imidazole (4.6 g, 67.5 mmol) and *tert*-butyldimethylsilyl chloride (4.88 g, 32.4 mmol) was added to a stirred solution of glycidol **255** (2 g, 27 mmol) in DMF (4 mL) at room temperature. The reaction mixture was stirred for 24 h. The mixture was diluted with water and extracted with ethyl acetate. The organic phase was dried and concentrated *in vacuo* to give an oil, which was vacuum distilled at 175 °C / 2 mm Hg to afford a thick oil, which tlc indicated to consist of two components. Further purification by column chromatography (hexane/acetone) afforded a yellow oil (0.5 g). The ^{1}Hnmr spectrum showed no trace of the desired product, but showed a strong presence of the silyl component.

Reaction of glycidol with *tert*-butyldimethylsilyl chloride, 4-dimethylaminopyridine and Et_3N in CH_2Cl_2.[241]

A mixture of 4-dimethylaminopyridine (0.13 g, 1.08 mmol), *tert*-butyldimethylsilyl chloride (4.48 g, 29.70 mmol) and Et_3N (3 g, 4.14 mL, 29.70 mmol) was stirred at room temperature under N_2 atmosphere. Glycidol **255** (2 g, 27 mmol) in CH_2Cl_2 (10 mL) was added to the mxiture and stirring continued for 24 h.

The mixture was washed with water, followed by saturated NH_4Cl, and dried. The solvent was removed *in vacuo* and product distilled at 75-80 °C / 2 mm Hg to give 1-*O*-*tert*-butyldimethylsilyl-2,3-epoxypropane **258** (4.22 g, 83 %) as a clear oil.



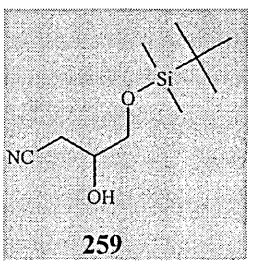
258

IR (neat) ν 2930, 1472, 1098 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 0.05 (6H, s, $Si(C\underline{H}_3)_2$), 0.8 (9H, s, $SiC(C\underline{H}_3)_3$), 2.55 (1H, dd, J=2.7, 5.2Hz, $C\underline{H}_2CH$-), 2.69 (1H, t, J=5Hz, $C\underline{H}_2CH$-), 2.98 -3.04 (1H, m, $CH_2C\underline{H}$-), 3.57 (1H, dd, J=4.8, 11.9Hz, -$C\underline{H}_2O$-), 3.77 (1H, dd, J=3.1, 11.9Hz, -$C\underline{H}_2O$-).

Reaction of 1-*O*-*tert*-butyldimethylsilyl-2,3-epoxypropane with KCN.[239]

A solution of 1-*O*-*tert*-butyldimethylsilyl-2,3-epoxypropane **258** (1 g, 5.31 mmol) in CH_3CN (10 mL) was treated with anhydrous lithium perchlorate (0.80 g, 7.50 mmol) and potassium cyanide (0.38 g, 5.84 mmol). The reaction mixture was stirred at 70 °C for 8 h and then cooled to room temperature. The mixture was diluted with water, extracted with ethyl acetate and dried. Removal of the solvent *in vacuo* followed by distillation at 125-130 °C / 3 mm Hg afforded 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile **259** (0.8 g, 70 %) as a clear yellow oil.



259

IR (neat) ν 3453, 2931, 2255, 1468, 1122 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 0.02 (6H, s, $Si(C\underline{H}_3)_2$), 0.85 (9H, s, $SiC(C\underline{H}_3)_3$), 2.5 (2H, m, -$C\underline{H}_2CN$), 2.72 (1H, d, -O$\underline{H}$), 3.7 (2H, m, -$C\underline{H}_2O$-), 3.9 (1H, m, C$\underline{H}$OH).

Attempted reaction of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile with phosphoric acid.[242]

4-(*Tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile **259** (0.25 g, 1.16 mmol) and 85 % phosphoric acid (1 mL) was heated in an oil bath to about 200 °C for 30 min. The reaction mixture was cooled to room temperature and neutralised with dilute NaOH solution. The mixture was extracted with ethyl acetate. The organic extracts were washed with brine, dried, and the solvent removed *in vacuo* to give a residue. The dark residue was vacuum distilled at 130-145 °C / 3 mm Hg to afford a

clear liquid, however, the peak of the nitrile group were absent from the IR spectrum, so the reaction abandoned.

Attempted reaction of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile with thionyl chloride.[243]

Thionyl chloride (3.3 g, 27.42 mmol) was added drop-wise to a stirred solution of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile **259** (0.05 g, 0.23 mmol) in pyridine (1 mL) at room temperature under N_2 atmosphere. The reaction mixture was stirred for 1 h and then quenched with ice water followed by extraction with ethyl acetate. The organic extracts were dried and concentrated *in vacuo*. The residue was vacuum distilled at 70-100 °C / 4 mm Hg to afford a small amount of a clear liquid. The IR spectrum for the clear liquid showed no indication of a nitrile group, therefore, the reaction was discontinued.

Attempted reaction of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile with phosphoryl chloride.[244]

A solution of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile **259** (0.7 g, 3.25 mmol) in dry pyridine (2 mL) was cooled to 0 °C and phosphoryl chloride (5 mL, 53.64 mmol) was added drop-wise. The reaction mixture was stirred at room temperature for 24 h, then diluted with water, followed by extraction with ether. The ether extracts were washed with dilute HCl solution, saturated $NaHCO_3$ solution, water, and then dried. The solvent removed *in vacuo* to afford a small amount of thick dark brown crude oil. The IR spectrum indicated the absence of a nitrile group, thus the reaction was abandoned.

Attempted reaction of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile with *p*-toluenesulphonic acid monohydrate.[245]

A solution of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile **259** (0.25 g, 1.16 mmol) with *p*-toluenesulphonic acid monohydrate (0.22g, 1.16 mmol) in dry toluene (60 mL) was refluxed for 2 h. The mixture was cooled to room temperature and washed with water. The toluene extracts were dried and concentrated

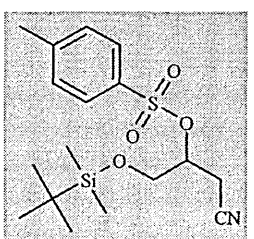
in vacuo to afford a very small amount of thick brown crude oil, which showed no sign of expected product in the IR spectrum and, therefore, the crude reaction mixture was discarded.

Attempted reaction of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile with *p*-toluenesulphonic acid adsorbed on silica gel.[246]

A solution of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile **259** (0.12 g, 0.56 mmol) in dry toluene (25 mL) was added to *p*-toluenesulphonic acid adsorbed on silica gel (2 g) and the mixture was stirred for 3 h under reflux. The reaction mixture was cooled to room temperature and petroleum spirit (30-50 °C, 25 mL) was added. The slushy mixture was purified by column chromatography (9:1 petrol/ethyl acetate) to give a yellow liquid which was vacuum distillated at 120-150 °C / 4 mm Hg to afford a clear liquid. The IR spectrum for the clear liquid revealed that the peak for the nitrile group was absent and, therefore, the liquid was discarded.

Reaction of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile with *p*-toluenesulphonyl chloride.[247]

Pyridine (0.2 g, 0.2 mL, 2.3 mmol) and *p*-toluenesulphonyl chloride (0.3 g, 1.7 mmol) was added to a stirred mixture of 4-(tert-Butyl-dimethyl-silanyloxy)-3-hydroxy-butyronitrile **259** (0.3 g, 1.2 mmol) in $CHCl_3$ (5 mL) at 0 °C. The reaction mixture was stirred at room temperature for 24 h. The mixture was diluted with water and extracted with ether. The organic extracts were washed with dilute HCl, saturated $NaHCO_3$ solution, water, and dried. The solvent was evaporated *in vacuo* to give a crude 4-(*tert*-butyldimethylsilanyloxy)-3-(toluene-4-sulphonyl)butyronitrile **260** (0.32 g, 75 %) as a white solid.



260

IR (KBr) ν 2926, 2855, 2254, 1463, 1377, 1120, 839 cm^{-1}.

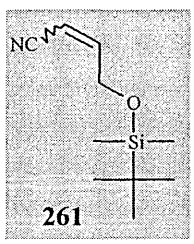
^{1}HNMR (300MHz, $CDCl_3$) δ 0.02 (6H, s, $Si(C\underline{H}_3)_2$), 0.89 (9H, s, $SiC(C\underline{H}_3)_3$), 2.52 (3H, s, Ar-$C\underline{H}_3$), 2.61 (2H, m, -$C\underline{H}_2CN$), 3.6 (2H, m, -$C\underline{H}_2O$-), 3.8 (1H, m, -C<u>H</u>-), 7.30, 7.91 (each 2H, d, Ar-<u>H</u>).

Attempted reaction of 4-(tert-butyldimethylsilanyloxy)-3-(toluene-4-sulphonyl)butyronitrile with potassium hydroxide.[248]

Potassium hydroxide (0.6 g, 10.69 mmol) in ethanol (15 mL) and 4-(*tert*-butyldimethylsilanyloxy)-3-(toluene-4-sulphonyl)butyronitrile **260** (0.28 g, 0.76 mmol) was stirred at room temperature for 1 h after which tlc showed the starting material remained. Therefore, the reaction mixture was warmed to 40 °C for 1 h and stirring continued at room temperature for 2 days. Random interval of tlc analysis revealed the starting material still remained and, therefore, the reaction mixture was abandoned.

Reaction of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile with methanesulphonyl chloride.[249]

A mixture of 4-dimethylaminopyridine (1 g, 8.7 mmol) and triethylamine (15 mL) in CH_2Cl_2 (20 mL), was maintained in low temperature with an ice-water bath. To the stirred mixture, a solution of 4-(*tert*-butyldimethylsilanyloxy)-3-hydroxybutyronitrile **259** (1.9 g, 8.7 mmol) in CH_2Cl_2 (20 mL) was added in dropwise. Methanesulphonyl chloride (2.5 g, 21.8 mmol) was added in dropwise with the temperature maintained between 10-20 °C and stirred for 1 h. The reaction mixture was stirred for a further 24 h at room temperature. The mixture was quenched with saturated $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic extracts were dried and concentrated *in vacuo*. The residue was purified by distillation to afford 4-(*tert*-butyldimethylsilanyloxy)-but-2-enenitrile **261** (0.4 g, 21 %) as a yellow oil.



261

IR (neat) ν 2929, 2858, 2225, 1642, 1472, 1379, 1134 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) E:Z (1:0.4)

E form δ 0.05 (6H, s, $Si(C\underline{H}_3)_2$), 0.81 (9H, s, $SiC(C\underline{H}_3)_3$), 4.28 (2H, dd, $-C\underline{H}_2O-$), 5.68 (1H, dt, J=16.3 Hz, $=C\underline{H}$), 6.78 (1H, dt, J= 16.3 Hz, $=C\underline{H}$).

Z form δ 0.05 (6H, s, $Si(C\underline{H}_3)_2$), 0.81 (9H, s, $SiC(C\underline{H}_3)_3$), 4.39 (2H, dd, $-C\underline{H}_2O-$), 5.30 (1H, dt, J=11.0 Hz, $=C\underline{H}$), 6.41 (1H, dt, J= 11.0 Hz, $=C\underline{H}$).

Attempted reaction of glycidol and benzyl bromide with sodium hydride in THF.[216]

Under N_2 atmosphere, the dry THF (100 mL) was added to sodium hydride (2.11 g, 52.8 mmol, 60 % dispersion in mineral oil) at 0 °C. Glycidol **255** (5 g, 67.5 mmol) in dry THF (50 mL) was added was to the suspended mixture and stirred for 30 min at room temperature. The mixture was cooled to 0 °C and benzyl chloride (11.11 g, 87.7 mmol) was added. After stirring for 4 h at room temperature, the mixture was quenched with saturated NH_4Cl solution and extracted with ethyl acetate. The combine extracts were washed with water, followed by brine, and dried. The solvent was removed *in vacuo* and the residue was vacuum distilled at 55-60 °C / 3 mm Hg to afford a clear liquid (8.05 g) which the ir and ^{1}Hnmr spectrum indicated to be dibenzyl ether.

IR (neat) ν 3066, 3033, 2962, 1455, 1073, 767, 698 cm^{-1}.

^{1}HNMR (300 MHz, $CDCl_3$) 4.58 (4H, s, -OC$\underline{H}_2$-Ar), 7.32 (10H, s, Ar-$\underline{H}$),

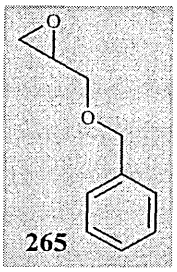
Attempted reaction of glycidol and benzyl chloride with sodium hydride in DMF.[216]

Under N_2 atmosphere, the dry DMF (100 mL) was added to sodium hydride (2.11 g, 52.8 mmol, 60 % dispersion in mineral oil) at 0 °C. Glycidol **255** (5 g, 67.5 mmol) in dry DMF (50 mL) was added to the suspended mixture and stirred for 30 min at room temperature. The mixture was cooled to 0 °C and benzyl chloride (11.11 g, 87.7 mmol) was added. After stirring for 12 h at room temperature, the mixture was quenched with saturated NH_4Cl solution and extracted with ethyl acetate. The combine extracts were washed with water, followed by brine, and dried. The solvent was removed *in vacuo* and to give a yellow residue, which the tlc and IR spectrum indicated to be dibenzyl ether.

Benzylation of gylcidol using benzyl bromide, sodium hydride with *tetra*-n-butylammonium iodide in THF.[250]

Under N_2 atmosphere, the dry THF (100 mL) was added to the sodium hydride (2.24 g, 56 mmol, 60 % dispersion in mineral oil) and *tetra*-n-butylammonium iodide (0.21 g, 0.57 mmol) at 0 °C. Glycidol **255** (4 g, 54 mmol) in dry THF (40 mL) was

added to the suspended mixture and stirred for 30 min. The mixture was cooled to 0 °C and benzyl bromide (9.54 g, 55.8 mmol) was added. After stirring for 1 h at room temperature, the mixture was quenched with saturated NH_4Cl solution and extracted with ethyl acetate. The combine extracts were washed with water, followed by brine, and dried. The solvent was removed *in vacuo* and the residue was subjected to column chromatography (5:1 petrol/ethyl acetate) to afford 2-benzyloxymethyloxirane **265** (3.90 g, 44 %) as a light yellow oil.



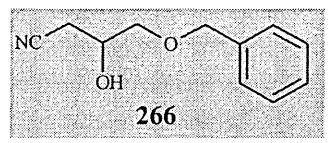
265

IR (neat) ν 3031, 2861, 1454, 1097, 739, 699 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 2.61 (1H, dd, J=2.7, 5Hz, C<u>H</u>$_2$CH-), 2.80 (1H, t, J=5Hz, C<u>H</u>$_2$CH-), 3.16 -3.22 (1H, m, -C<u>H</u>-), 3.42 (1H, dd, J=5.9, 11.4Hz, -C<u>H</u>$_2$O-), 3.77 (1H, dd, J=3, 11.4Hz, -C<u>H</u>$_2$O-), 4.54 & 4.61 (each 1H, d, J=12Hz, -OC<u>H</u>$_2$-Ar), 7.24 -7.37 (5H, m, Ar-<u>H</u>).

<u>Reaction of 2-benzyloxymethyloxirane with KCN.</u>[239]

A solution of 2-benzyloxymethyloxirane **265** (3.50 g, 21.31 mmol) in CH_3CN (80 mL) was treated with anhydrous lithium perchlorate (3.40 g, 31.97 mmol) and potassium cyanide (2.08 g, 31.97 mmol). The reaction mixture was stirred at 70 °C for 24 h in dry atmosphere and then cooled to room temperature. The mixture was diluted with water, extracted with ethyl acetate and dried. The solvent was removed *in vacuo* to afford the crude 4-benzyloxy-3-hydroxybutyronitrile **266** (3.46 g, 85 %) as a brown oil.



266

IR (neat) ν 3444, 3032, 2926, 2255, 1454, 1113, 741, 700 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 2.58 (2H, m, -C<u>H</u>$_2$CN), 2.99 (1H, m, -O<u>H</u>), 3.50 (2H, m, -C<u>H</u>$_2$O-), 4.05 (1H, m, C<u>H</u>OH), 4.50 (2H, dd, -OC<u>H</u>$_2$-Ar), 7.24-7.40 (5H, m, Ar-<u>H</u>).

<u>Attempted reaction of 4-benzyloxy-3-hydroxybutyronitrile with sodium hydroxide in ethanol.</u>

Sodium hydroxide (1 g, 25 mmol) in ethanol (10 mL) was added to the stirred solution of 4-benzyloxy-3-hydroxybutyronitrile **266** (0.5 g, 2.62 mmol) in ethanol (10 mL). The reaction mixture was stirred at 40 °C for 6 h. Diluted hydrochloric acid solution was added to neutralise the reaction mixture and then the mixture was

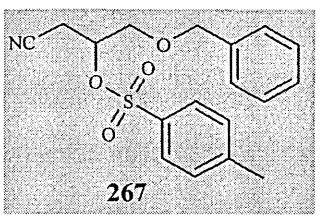
extracted with CH_2Cl_2. The organic extracts were dried and concentrated *in vacuo* to afford a brown oil (0.52 g). The IR spectrum for the brown oil revealed that the starting material still remained and, therefore, the reaction was discontinued.

Attempted reaction of 4-benzyloxy-3-hydroxybutyronitrile with phosphoric acid.[242]

4-Benzyloxy-3-hydroxybutyronitrile **266** (0.5 g, 2.62 mmol) and 85 % phosphoric acid (40 mL) was refluxed for 30 min. The reaction mixture was cooled to room temperature and neutralised with dilute NaOH solution. The mixture was extracted with ether. The organic extracts were washed with brine and dried. The solvent was removed *in vacuo* to give a dark residue. The IR spectrum of the dark residue indicated the absence of a nitrile group, thus, the reaction was abandoned.

Tosylation of 4-benzyloxy-3-hydroxybutyronitrile.[247]

Pyridine (0.8 g, 0.8 mL, 10.5 mmol) and *p*-toluenesulphonyl chloride (1.5 g, 7.8 mmol) was added to a stirred mixture of 4-benzyloxy-3-hydroxy-butyronitrile **266** (1 g, 5.2 mmol) in $CHCl_3$ at 0 °C. The reaction mixture was stirred at room temperature for 24 h. The mixture was diluted with water and extracted with ether. The organic extracts were washed with dilute HCl, saturated $NaHCO_3$ solution, water, and then dried. The solvent was evaporated *in vacuo* to give a crude 4-benzyloxy-3-(toluene-4-sulphonyloxy)butyronitrile **267** (1.42 g, 77 %) as a white semi-solid.



267

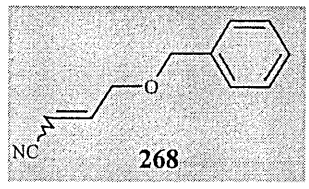
IR (neat) ν 3091, 3061, 2925, 2254, 1455, 1373, 1121, 814, 702, 751cm^{-1}.

Synthesis of 4-benzyloxybut-2-enenitrile.

Reaction of 4-benzyloxy-3-(toluene-4-sulphonyloxy)butyronitrile with triethylamine.[249]

4-Benzyloxy-3-(toluene-4-sulphonyloxy)butyronitrile **267** (1.8 g, 5.2 mmol) and triethylamine (2 mL) in CH_2Cl_2 (25 mL) was stirred at room temperature for 24 h. The reaction mixture was concentrated to a dark residue and purified by column

chromatography (60-80 % ethyl acetate/petrol) to give 4-benzyloxybut-2-enenitrile **268** (0.7 g, 77 %) as a brown oil.



A small fraction was further purified by kugelröhr distillation to afford yellow oil for analysis.

IR (neat) ν 3032, 2870, 2223, 1670, 1636, 1456, 1334, 1155, 1116, 738, 698 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) E:Z (1.5:1)

E form δ 4.14 (2H, dd, J= 1.2 and 2.4 Hz, -C$\underline{H}_2$O-), 4.56 (2H, s, -OC$\underline{H}_2$-Ar), 5.72 (1H, dt, J=16.3 and 2.4 Hz, =C$\underline{H}$), 6.74 (1H, dt, J=16.3 and 2.4 Hz, =C$\underline{H}$), 7.36 (5H, m, Ar-$\underline{H}$).

Z form δ 4.35 (2H, dd, J= 1.7 and 4.3 Hz, -C$\underline{H}_2$O-), 4.56 (2H, s, -OC$\underline{H}_2$-Ar), 5.46 (1H, dt, J=11.3 and 1.7 Hz, =C$\underline{H}$), 6.61 (1H, dt, J=11.3 and 1.7 Hz, =C$\underline{H}$), 7.36 (5H, m, Ar-$\underline{H}$).

Reaction of 4-benzyloxy-3-hydroxybutyronitrile with methanesulphonyl chloride.[249]

A solution of 4-benzyloxy-3-hydroxybutyronitrile **266** (5.5 g, 28.8 mmol) in CH_2Cl_2 (20 mL) was added to a stirred mixture of 4-dimethylaminopyridine (3.5 g, 28.7 mmol) and triethylamine (35 mL) in CH_2Cl_2 (20 mL), at 5-10 °C. Methanesulphonyl chloride (16.5 g, 143.8 mmol) was added with the temperature maintained between 10-20 °C and stirred for 1 h. The reaction mixture was stirred for a further 24 h at room temperature. The mixture was quenched with saturated $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic extracts were dried and concentrated *in vacuo*. The residue was subjected to column chromatography (70 % ethyl acetate/petrol) to afford 4-benzyloxybut-2-enenitrile **268** (4.82 g, 97 %) as brown oil.

A small fraction was further purified by kugelröhr distillation to afford yellow oil for analysis.

IR (neat) ν 3033, 2859, 2224, 1668, 1640, 1455, 1116, 1028, 738, 699 cm^{-1}.

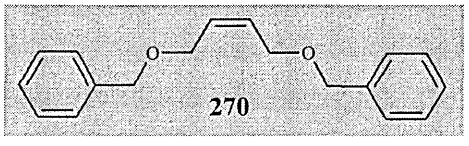
^{1}HNMR (300MHz, $CDCl_3$) E:Z (2:1)

E form δ 4.16 (2H, dd, J= 1.2 and 2.3 Hz, -C$\underline{H}_2$O-), 4.57 (2H, s, -OC$\underline{H}_2$-Ar), 5.75 (1H, dt, J=16.2 and 2.3 Hz, =C$\underline{H}$), 6.76 (1H, dt, J=16.2 and 2.3 Hz, =C$\underline{H}$), 7.32 (5H, m, Ar-$\underline{H}$).

Z form δ 4.36 (2H, dd, J=1.7 and 4.2 Hz, -C<u>H</u>$_2$O-), 4.57 (2H, s, -OC<u>H</u>$_2$-Ar), 5.48 (1H, dt, J=11.3 and 1.7 Hz, =C<u>H</u>), 6.62 (1H, dt, J=11.3 and 1.7 Hz, =C<u>H</u>), 7.32 (5H, m, Ar-<u>H</u>).

<u>Benzylation of 2-butene-1,4-diol with benzyl bromide.</u>[250]

Dry DMF (100 mL) was added to a stirred mixture of sodium hydride (4.54 g, 113.49 mmol, 60 % dispersion in mineral oil) and *tetra*-n-butylammonium iodide (0.21 g, 0.567 mmol), which was cooled in an ice-water bath under N_2 dropwise atmosphere. To the suspension, 2-butene-1,4-diol **269** (5 g, 56.75 mmol) was added with the temperature maintained between 5-10 °C. Then benzyl bromide (19.41 g, 113.49 mmol) was added dropwise with the temperature maintained below 40 °C. The reaction mixture was stirred for a further 24 h at room temperature. The mixture was cooled and diluted with water (50 mL), followed by extraction with 1:1 Et_2O/hexane (3 x ~50 mL). The combined extracts were dried and concentrated *in vacuo* to give crude 1,4-dibenzyloxy-2-butene **270** (13.71 g, 90 %) as a light yellow oil.



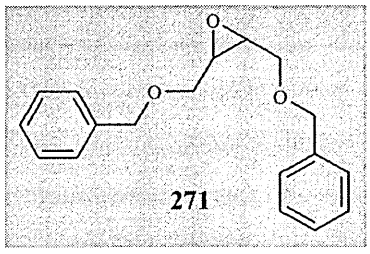
270

IR (neat) ν 3030, 2855, 1455, 1091, 737, 698 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 4.03 (4H, dd, -C<u>H</u>$_2$O-), 4.46 (4H, s, -OC<u>H</u>$_2$-Ar), 5.77 (2H, dt, J=3.7 and 1.0Hz, <u>H</u>C=C<u>H</u>), 7.31 (10H, m, Ar-<u>H</u>).

<u>Epoxidation of 1,4-dibenzyloxy-2-butene with m-chloroperbenzoic acid.</u>[250]

1,4-Dibenzyloxy-2-butene **270** (13.25 g, 49.38 mmol) in CH_2Cl_2 (175 mL) was placed in a 500 mL round-bottom flask equipped with a dropping funnel and $CaCl_2$ drying tube. A solution of *m*-chloroperbenzoic acid (34.09 g, 197.54 mmol, 50 % pure) in CH_2Cl_2 (175 mL) was slowly added. The mixture was stirred at room temperature for 24 h. Saturated sodium metabisulphite solution was added to the mixture (to destroy the peracid) followed by saturated $NaHCO_3$ solution, and extracted with ether. The organic extracts were washed with brine, water, and dried. The solvent was removed *in vacuo* to give crude 1,4-benzyloxy-2,3-epoxybutane **271** (13.51 g, 96 %) as an oily white solid.



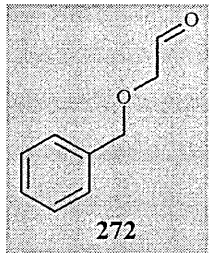
271

IR (neat) ν 3064, 3031, 2859, 1454, 1096, 738, 698 cm^{-1}.

^{1}HNMR (300MHz, $CDCl_3$) δ 3.26 (2H, m, -C<u>H</u>-C<u>H</u>-), 3.53 (2H, dd, J=6.5 and 11.3Hz, -C<u>H</u>$_2$O-), 3.69 (2H, dd, J=3.9 and 11.3Hz, -C<u>H</u>$_2$O-), 4.51 (2H, d, J=11.9 Hz, -OC<u>H</u>$_2$-Ar), 4.61 (2H, d, J=11.9Hz, -OC<u>H</u>$_2$-Ar), 7.29-7.38 (10H, m, Ar-<u>H</u>).

<u>Oxidative cleavage of 1,4-benzyloxy-2,3-epoxybutane with periodic acid.</u>[250]

1,4-Benzyloxy-2,3-epoxybutane **271** (5 g, 17.58 mmol) in THF (100 mL) was stirred at room temperature and periodic acid (4 g, 17.58 mmol) was added in one portion to this mixture. The resulting yellow solution was stirred for 24 h. The reaction mixture was washed with water and extracted with ether. The combined layers were washed with water, brine, and dried. The solvent removed *in vacuo* gave a yellow semi-solid crude (11.59 g) which IR analysis revealed to be 2-benzyloxyacetaldehyde **272**. The crude was used without further purification.



272

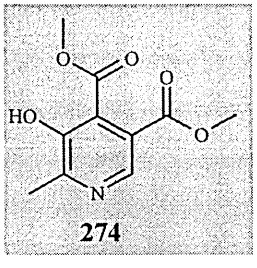
IR (neat) ν 2924, 1727, 1454, 1428, 1095, 736, 699 cm^{-1}.

<u>Attempted Wittig-Horner reaction of 2-benzyloxyacetaldehyde with diethyl cyanomethanephosphonate.</u>[252]

A solution of diethyl cyanomethanephosphonate (1.96 g, 11.06 mmol) in THF (10 mL) was added to a stirred suspension of powdered potassium hydroxide (1.24 g, 22. 11 mmol) in THF (10 mL) at 0 °C. 2-Benzyloxyacetaldehyde **272** (1.7 g, 11.06 mmol) in THF (10 mL) was added to the reaction mixture at 0 °C. The reaction mixture was stirred at room temperature for 12 h. The mixture was washed with water and extracted with ether. The organic extracts were dried and concentrated *in vacuo* to afford a dark residue. The IR spectrum of the residue revealed that the peak for the nitrile group was absent and, therefore, the reaction was abandoned.

<u>Diels-Alder reaction of 4-methyl-5-ethoxyoxazole with dimethyl maleate.</u>[253]

4-Methyl-5-ethoxyoxazole **222** (0.7 g, 5.1 mmol) and dimethyl maleate **273** (1.4 g, 10 mmol) in toluene (10 mL) was refluxed for 24 h. The reaction mixture was concentrated to a residue and purified by



274

column chromatography ($CHCl_3$) to give dimethyl 5-hydroxypyridine-3,4-dicarboxylate **274** (0.4 g, 36 %) as a cream solid with mp 128-132 °C (lit mp 140-141 °C[253]).

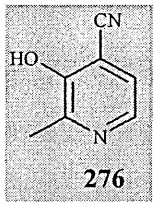
IR (KBr) ν 3358, 2956, 1744, 1440 cm^{-1}.

^{1}HNMR (60MHz, $CDCl_3$) δ 2.57 (3H, s, 2-C<u>H</u>$_3$), 3.90 (3H, s, -CO_2C<u>H</u>$_3$), 3.94 (3H, s, -CO_2C<u>H</u>$_3$), 8.26 (1H, s, 6-<u>H</u>).

MS [m/z (relative intensity)] 225 (M^+, 33), 193 (100).

<u>Diels-Alder reaction of 4-methyl-5-ethoxyoxazole with acrylonitrile.</u>[254]

4-Methyl-5-ethoxyoxazole **222** (0.65 g, 5.1 mmol) and acrylonitrile **275** (10 mL) was stirred at room temperature for 24 h. The excess acrylonitrile was removed *in vacuo* and the residue was crystallised from methanol to give 4-cyano-3-hydroxy-2-methylpyridine **276** (0.23 g, 34%) as a light orange solid with mp 235-240 °C (lit mp 235-237 °C[254]).



276

IR (KBr) ν 2223.5 cm^{-1}.

^{1}HNMR (300MHz, DMSO-d_6) δ 2.45 (3H, s, py-C<u>H</u>$_3$), 7.48 (1H, d, J=5 Hz, py-<u>H</u>), 8.00 (1H, d, J=5 Hz, py-<u>H</u>).

MS [m/z (relative intensity)] 134 (M^+, 100).

<u>Attempted Diels-Alder reaction of 4-methyl-5-ethoxyoxazole with 4-benzyloxy-but-2-enenitrile.</u>[232]

4-Methyl-5-ethoxyoxazole **222** (0.6 g, 4.96 mmol), 4-benzyloxybut-2-enenitrile **268** (0.8 g, 4.23 mmol) and hydroquinone (0.05 g) in methanol (15 mL) was refluxed for 24 h. The reaction mixture was kept in the fridge for 24 h, followed by addition of ethanol saturated with hydrochloride gas (25 mL). The mixture was stand for 3 h on ice and then acetone (10 mL) was added to precipitates the salt. However, no precipitation occurred and the tlc showed the present of the oxazole, therefore, the reaction was discontinued.

5. ***References***

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